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Volume 154, Number 1, January 1977

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Proceedings of The Society for Experimental Biology and Medicine, Vol. 154, No. 1, January, 1977. Published monthly except August by Academic Press, Inc., 111 Fifth Avenue, New York, N. Y. 10003. Second class postage paid at New York, N. Y. and at additional mailing offices. Subscription per year $45.00 U.S.A.: $51.50 outside U.S.A. (plus postage). Send notices of change of address to the Office of the Publisher at least 6–8 weeks in advance. Please include both the old and new addresses. Copyright © 1977 by the Society for Experimental Biology and Medicine.

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I OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 1-5 (1977)

ect of Age on Hyperphagia in the Genetically Obese Zucker Rat[1,2] (39589)

BARBARA A. DILETTUSO AND PAUL J. WANGSNESS

ent of Dairy and Animal Science, The Pennsylvania State University, University Park, Pennsylvania 16802

ie animals, such as the genetically Zucker rat (1-3), consume excessive ts of food as compared to lean litter- Obese rats also weigh more than lean), so it is reasonable that obese rats ie more total food per day. Extrapo- rom several studies utilizing mature -4), however, revealed that when take was expressed per unit of body (FI/BW), obese rats were not hyper-

Because several reports indicated etabolic activity was elevated in the rapidly growing obese rat (5, 6), we ted that hyperphagia in terms of in- FI/BW would most likely occur in rats. The objective of the present /as to determine the effect of age on in lean and obese Zucker rats. Our demonstrated that hyperphagia was in the young obese rat but disap- in the older animal.

rials and methods. Experiment 1. an rats and 12 obese rats of the strain were individually caged and ned at 22 ± 1°. A commercial diet[3])und and supplemented with 1% oil the following nutrient composition: protein, 5.5% fat, 56.5% carbohy-).0% fiber, and 9.0% ash. Food and /ere offered *ad libitum*. Food intake is measured daily and body weight /as recorded on alternate days from 7 eeks of age. Weekly averages for FI / were calculated. At 7 weeks of age, iot difficult to determine which rats bese on the basis of FI, BW, and nation.

riment 2. Thirty-three Zucker rats from four complete litters were weaned and placed in individual cages maintained at 22 ± 1°. A semipurified diet (Table I) and water were offered *ad libitum*. This powdered diet consisted of 22.9% protein, 4.0% fat, 65.0% carbohydrate, 1.9% fiber, 4.0% ash, and 2.2% vitamin mixture. FI was measured daily and BW was recorded every 2 days from 3 to 11 weeks of age. BW and FI differences at 3.5 weeks of age confirmed our tentative visual identification of 26 lean and 7 obese rats when the trial was initiated. Weekly averages for FI, BW, and FI/BW for both experiments were compared by the unpaired Student's *t* test.

Results. Obese rats were observed to have a higher FI per day and BW than lean rats ($P < 0.01$) throughout Experiment 1 (Fig. 1). A faster rate of gain was apparent in the obese rats. Hyperphagia, defined as a higher FI/BW, was evident only up to 10 weeks of age ($P < 0.01$). From 13 to 18 weeks of age, there were no differences in FI/BW. At 19 weeks of age, obese rats were hypophagic compared to lean rats ($P < 0.01$).

TABLE I. COMPOSITION OF DIET USED IN EXPERIMENT 2 WITH WEANLING RATS.

Ingredient	%
Casein	22.5
DL-Methionine	0.4
Peanut oil	4.0
Corn starch	39.0
Glucose	19.5
Sucrose	6.5
Cellulose (Alphacel)	1.9
Vitamin mixture[a]	2.2
Mineral mixture[b]	4.0
	100.0

[a] Vitamin Diet Fortification Mixture; Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

[b] Jones and Foster Salt Mixture; Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

work was supported in part by NIH Biomedi- ces Support Grant No. 5505RR07082-08 to and NIH No. HD 07090-03 to R. J. M.

iorized for publication on November 20, 1975 No. 4975 in the journal series of the Pennsyl- ricultural Experiment Station.

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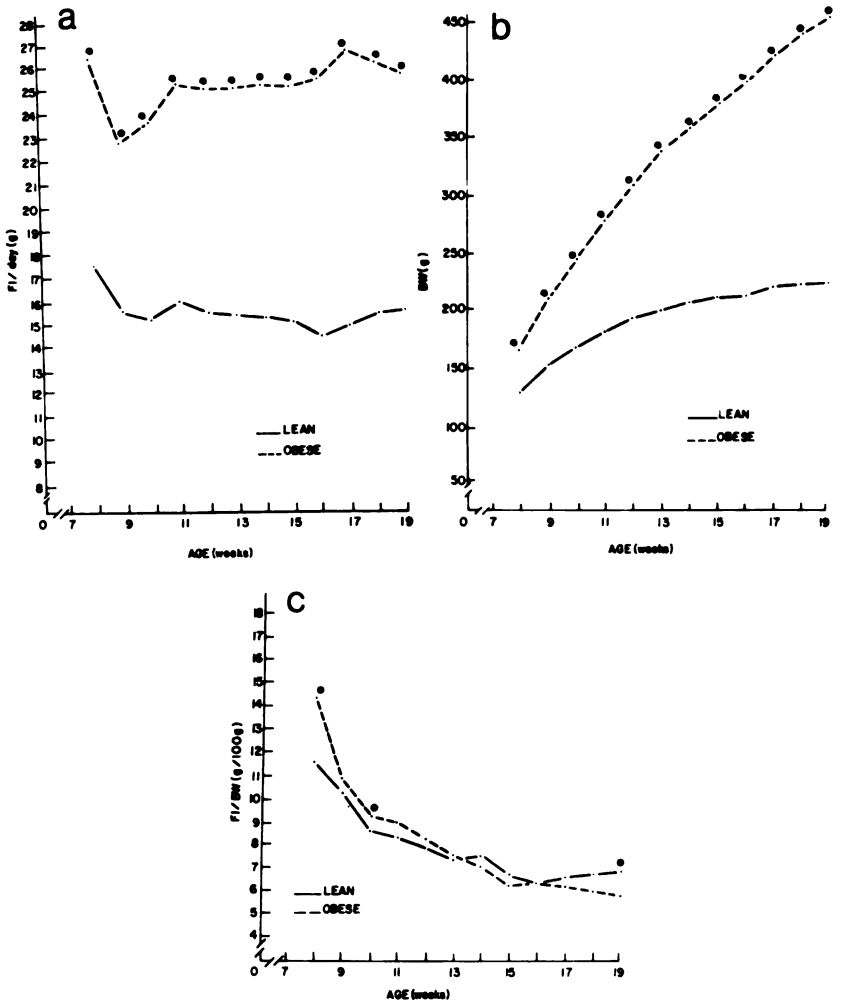
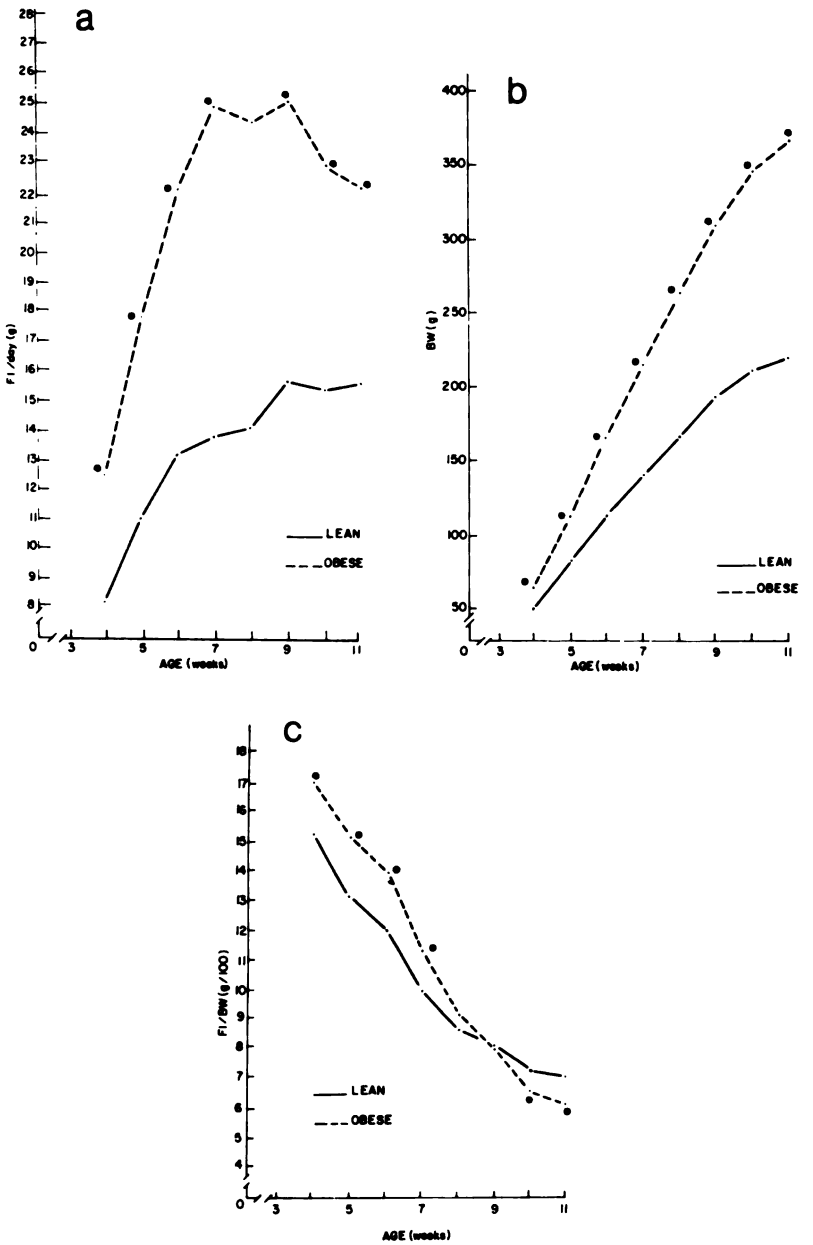


FIG. 1. (a) Food intake of lean and obese Zucker rats, 7 to 19 weeks of age. (b) Body weight of lean and obese Zucker rats, 7 to 19 weeks of age. (c) Food intake per 100 g of body weight of lean and obese Zucker rats, 7 to 19 weeks of age. * Significantly different from lean value ($P < 0.01$).

In Experiment 2 (Fig. 2), both total daily FI and BW were elevated in obese rats ($P < 0.01$) as was observed in Experiment 1. However, at this younger age, total food intake was increasing during the first 3 weeks of the experiment in both lean and obese groups, as compared to the relatively constant food intake in Experiment 1 with older rats. Daily FI leveled off in lean and obese rats at about 7 weeks of age to approximately the same FI observed at the initiation of Experiment 1 at 7 weeks of age. Rate of weight gain also appeared to be more rapid in the young lean and obese rats. FI/BW was higher in obese rats from 3 to 7 weeks of age ($P < 0.01$). There was no difference in FI/BW at 8 and 9 weeks of age and, thereafter, obese rats had lower FI/BW ($P < 0.01$).

When food intake was expressed on the basis of metabolic size ($BW^{0.75}$), similar relationships as those shown in Figs. 1 and 2 were observed. However, when $BW^{0.75}$ was used, the degree of hyperphagia of obese rats was even greater than when $BW^{1.0}$ was used.

Discussion. These experiments demonstrate that designating obese animals as hyperphagic, solely on the basis of excessive daily FI, is inappropriate. It has been shown



(a) Food intake of lean and obese Zucker rats, 3 to 11 weeks of age. (b) Body weight of lean and obese ts, 3 to 11 weeks of age. (c) Food intake per 100 g of body weight of lean and obese Zucker rats, 3 to 11 ıge. * Significantly different from lean value ($P < 0.01$).

that FI per day is greater in the Zucker obese rat as compared to the lean littermates (1, 2). When FI is expressed per unit of BW to compensate for large differences in BW, it is clear from our studies that obese rats were hyperphagic only from 3 to 7 weeks of age. Recalculation of data from several reports involving rats 6 to 12 weeks of age indicated no difference in FI/BW between lean or obese rats (2–4); 10- to 12-week-old obese rats had a lower FI/BW than leans (2). These results are in agreement with the present study since no difference in FI/BW was seen in rats from 7 to 10 weeks of age, and beyond 10 weeks of age, as the rate of body weight gain started to plateau, the obese rats were hypophagic compared to the lean rats.

The increased gain in body fat relative to protein with increasing age is a well-known phenomenon. In addition, rate of body gain generally decreases with age. Collectively, these observations could account for the general decrease in FI/BW observed in both the lean and obese animals with age in our studies. However, the differences in these age-related changes between lean and obese animals are not yet well defined. Inactivity in the older obese rat may also account for part of the difference in the FI/BW changes between lean and obese rats observed in our studies. Young obese Zucker rats have been shown to be less active and to have a lower basal metabolism than lean littermates (7). Also, older obese rats have been reported to be more sluggish and to have a lower maintenance requirement (2) than lean rats.

The age at which hyperphagia disappeared in the two experiments reported was not the same. In Experiment 1, hyperphagia ceased at about 10 weeks of age whereas it ended at about 7 weeks of age in Experiment 2 with the weanling rats. Rats in Experiment 2 grew faster and were approximately 25% heavier than those of Experiment 1 at 8 weeks of age. The semipurified diet used in Experiment 2 was lower in fiber and probably more palatable and more digestible than the chow diet used in Experiment 1. This probably enhanced maximal FI and enabled the weanling rats in Experiment 2 to grow faster, and thus allowed the obese rats to reach a set point of body energy balance (8) with a shorter period of hyperphagia.

The hyperphagia observed in early life would be consistent with the elevated lipid deposition, glucose metabolism, lipogenesis, and lipolysis observed in weanling obese rats (5, 6, 9). An overall increase in energetic efficiency during this early stage of development in the obese rat may be related to the observed hyperphagia. Obese rats, however, still retained significantly more energy as fat compared to lean rats (3, 10, 11) when food intake was restricted. These studies suggest that it would be advantageous to study metabolism as related to the control of food intake in the obese animal at an early age when hyperphagia is most evident.

Summary. Two experiments were conducted to determine if and when hyperphagia occurs in the genetically obese Zucker rat. In Experiment 1, lean and obese rats were offered food *ad libitum* from 7 to 19 weeks of age. Obese rats consumed significantly more total food than lean rats throughout the experiment. However, when food intake (FI) was expressed per unit of body weight (FI/BW) to compensate for the large differences in body weight between lean and obese rats, hyperphagia in the obese rats was only evident from 7 to 10 weeks of age. Thereafter, FI/BW became similar and at 19 weeks of age, FI/BW was significantly lower for obese rats compared to leans. Experiment 2 studied younger rats (3 to 11 weeks of age) and produced similar results. However, the increased FI/BW in obese rats was more pronounced at the early age and was evident from 3 to 7 weeks of age. It is concluded that obese rats were hyperphagic compared to lean rats, but only at an early age.

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Received April 8, 1976. P.S.E.B.M. 1977, Vol. 154

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 6–8 (1977)

Plasma Pseudorenin in Rats after Alteration in the Renin-Angiotensin System[1] (39590)

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An apparently new enzyme exhibiting renin-like activity but having a lower pH optimum has been described and partially characterized by Skeggs *et al.* (1), who conveniently refer to this enzyme as pseudorenin. Pseudorenin has been reported to occur in various tissues in the rat, but unlike renin it is not concentrated in the kidney (1). Pseudorenin activity is also present in the plasma of rats and humans. Preliminary studies showed no correlation between plasma pseudorenin and blood pressure in normal controls and in hypertensive patients (1). Since nothing is known concerning the factors which regulate plasma pseudorenin, we have initiated a study to investigate possible regulatory mechanisms. Initially, we utilized surgical and pharmacological interventions known to alter plasma renin activity. Both renin and pseudorenin levels in plasma were monitored in rats after nephrectomy or β-adrenergic blockade, which lower plasma renin (2–4), and after inhibition of converting enzyme, which has been shown to increase plasma renin (5).

Methods. Male Wistar rats (300–500 g) were anesthetized with pentobarbital sodium (50 mg/kg ip) and the left carotid artery and right jugular vein were cannulated with PE 50 tubing. In four rats, bilateral nephrectomy was performed and 0.3-ml arterial blood samples were collected immediately after nephrectomy and at 30-min intervals up to 130 min. A final sample was obtained at 180 min after nephrectomy. Samples were collected in small tubes containing 15 μl of 300 m*M* EDTA as an anticoagulant and centrifuged for 15 min to yield plasma. Fluid volume was replaced with 0.9% NaCl after each collection.

In four rats, propranolol (Inderal HCl, injectable), 1 mg/kg, was injected iv at zero time and a supplemental dose of 1 mg/kg was given at 30 min after the first injection. Blood samples were obtained as described above at 0, 30, and 60 min, and blood volume was replaced with 0.9% saline. Blood samples were obtained from four control rats following the schedule outlined for the nephrectomized group.

For the experiment utilizing the converting enzyme inhibitor SQ 20881 (CEI), two rats were catheterized and both kidneys of one rat were removed. Two hours after nephrectomy, 400 μg of CEI was injected iv into each rat followed by 400 μg im. Arterial blood samples (0.3 ml) were collected at 0, 5, 10, 20, 40, and 80 min after CEI injection. Volume was replaced with 0.9% NaCl.

Plasma angiotensin generation was measured by the radioimmunoassay technique of Haber *et al.* (6). For renin-dependent angiotensin generation, 30 μl of plasma was incubated for 2 hr at pH 7.0 with bovine serum substrate (500 pmoles/ml) in the presence of angiotensinase inhibitors (BAL, EDTA, and 8-hydroxyquinoline). Bovine substrate was prepared by the method of Lever *et al.* (7). Pseudorenin-dependent angiotensin generation was obtained by incubating 10-μl plasma samples with tetradecapeptide substrate (500 pmoles/ml) at pH 4.5 for 1 hr. The tetradecapeptide substrate was obtained from Schwarz/Mann. Angiotensin was assayed by radioimmunoassay using components from New England Nuclear. Enzyme assays were linear within the range utilized. Results were calculated as nanograms per milliliter per hour of angiotensin generated. For comparison, results are graphically expressed as percentage of the zero-time sample activity.

Results. The effect of bilateral nephrectomy on both pseudorenin and renin levels in the rat is shown in Fig. 1. In the nonoper-

[1] Supported by grants from the Kansas Heart Association, the American Heart Association, and NIH Grant No. HL 16102.

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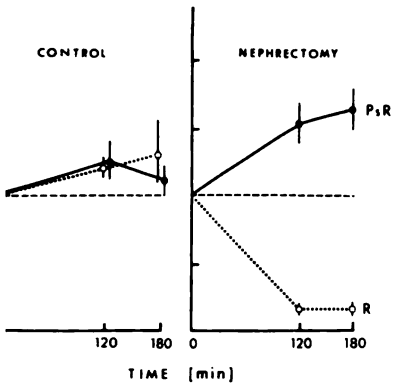

Effect of bilateral nephrectomy on plasma pseudorenin. (●——●) PsR, pseudorenin; R, renin; both are expressed as percentage ıe value. Each point represents mean ± SE ts. Zero-time renin and pseudorenin levels ± 3.3 ng/ml/hr of angiotensin and 393 ± 79 f angiotensin, respectively.

trols, plasma pseudorenin and renin ɔear to increase slightly with time, ı the only significant increase com- ith zero time occurs in the renin 120 min ($P < 0.05$). This apparent in plasma renin is probably associ- h an increased renin release in re- o hemorrhage, as has been demon- previously (8, 9). After nephrec- ːnin levels fell to 20 ± 5% of the e and remained at this level at 3 hr, pseudorenin significantly increased ıe zero time to 165 ± 17% at 180 < 0.05) (Fig. 1). These data clearly a nonrenal source for plasma pseu-

s pretreated with propranolol, re- nilar to those after nephrectomy ıtained. Sixty minutes after initial ɔlol treatment, renin plasma levels) ± 9% of the zero-time value (Fig. ıdorenin levels increased signifi- ı 127 ± 11% of zero time at 30 min .05), but at 60 min there was no nt increase. These data indicate that ːnin release is not under tonic β- ɡic influence unlike that demon- or renin release (8, 9). Preliminary our laboratory has also suggested ıdorenin is not released in response energic or cholinergic stimulation, more experiments are needed to is.

Since plasma pseudorenin levels tended to increase with a decrease in plasma renin, we decided to see if pseudorenin release is under feedback control of angiotensin II as has been demonstrated for renin release (3, 10–12). In the rat with functional kidneys, CEI treatment caused plasma renin levels to increase to 350% of the zero-time level 20 min after treatment, whereas pseudorenin levels failed to change significantly from the zero-time level (Fig. 3). In the nephrectomized rat, renin levels were at less than 10% of the zero-time level of the nonnephrectomized rat when CEI was injected. As expected, renin levels did not increase at any time after CEI treatment. Pseudorenin again did not change significantly from the zero time (Fig. 3).

Discussion. Evidence presented here demonstrates further the presence of an enzyme in rat plasma which exhibits renin-like activity *in vitro* first described by Skeggs *et al.* (1).

Although both renin and pseudorenin will generate angiotensin *in vitro*, this probably does not occur in plasma with pseudorenin *in vivo* since it is inactive on plasma substrate at physiological pH (1). Lack of response of plasma pseudorenin levels to alterations in plasma renin produced by nephrectomy, β-blockade, or converting enzyme inhibition, also suggests that pseudorenin is not involved in the renin-angiotensin system. The physiological role of circu-

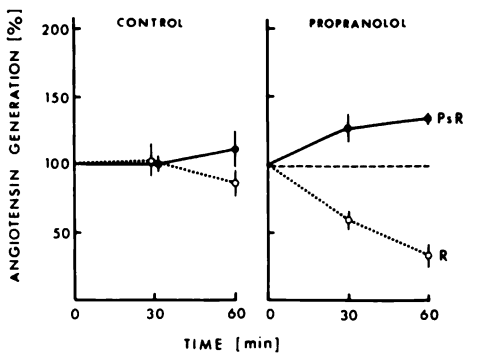

FIG. 2. Effect of propranolol pretreatment on plasma renin and pseudorenin. (●——●) PsR, pseudorenin; (O-----O) R, renin; both are expressed as percentage of zero-time activity. Each point represents mean ± SE of four rats. Zero-time renin and pseudorenin levels were 28.7 ± 6.0 ng/ml/hr of angiotensin and 470 ± 71 ng/ml/hr of angiotensin, respectively.

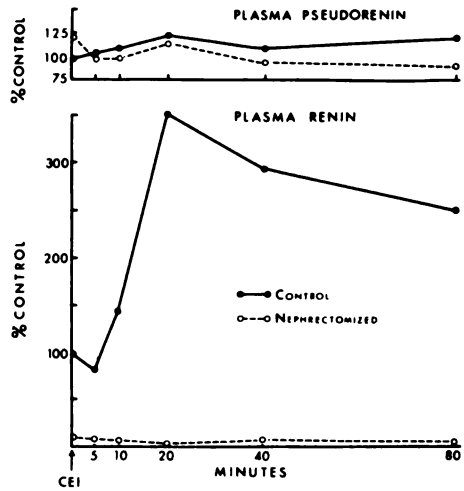

FIG. 3. Effect of treatment with SQ 20881 (400 μg, iv) on plasma pseudorenin and renin levels in a normal and a nephrectomized rat. (●——●) Control; (O-----O) nephrectomized. Top portion represents pseudorenin values expressed as percentage of the zero-time activity of the nonnephrectomized rat. Lower portion shows renin levels expressed as percentage of the zero-time activity in the nonnephrectomized rat.

lating pseudorenin, if one exists, is not known. More investigation into the site of production and physiological mechanisms which govern formation and release of pseudorenin is needed before any conclusions concerning its physiological role can be drawn.

Summary. Plasma levels of renin and pseudorenin were measured at various times in control rats, and after bilateral nephrectomy, treatment with propranolol, or the converting enzyme inhibitor (CEI) SQ 20881. After nephrectomy or propranolol, renin levels decreased but pseudorenin levels increased. Following treatment with CEI, renin levels rose in a nonoperated animal and remained very low in a nephrectomized animal. Pseudorenin levels did not change appreciably after CEI in either animal.

We acknowledge the valuable technical assistance of Mr. John Gillespie and Mrs. Roselle Poisner. We wish to thank E. R. Squibb for the generous supply of SQ 20881.

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Received June 1, 1976, P.S.E.B.M. 1977, Vol. 154.

)F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 9-13 (1977)

Border Antibodies of Heymann Nephritis of Rats in Normal Human Serum (39591)

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ann and associates (1) in 1959 orted the experimental production irotic syndrome in rats by repeated s of a mixture containing rat kidney ind complete Freund's adjuvant This disease subsequently has been o be an immunologically induced nous glomerulonephropathy and is histologically and immunohistolog- the idiopathic membranous glomer- opathy (IMGN) in humans (2–4). unized rats produce autoantibodies il renal tubular antigens, (5, 6) and ie main disease-producing autoanti- eems to be the brush border anti- BAb) (2, 5, 7). This antibody is against an antigen (BBAg) present ush border region of the epithelial the proximal renal tubules (2). as been recovered from rat kidneys oluble, large molecular weight lipo- (7), and is also present in small in the circulation of normal rats n immunized rat, it (BBAg) com- ith its autoantibody (BBAb) to able immune complexes, which are pped in the glomerular capillary oduce immune injury, and lead to lopment of glomerulonephropathy. tiology and pathogenesis of the n humans is not known. Japanese tors (8) have reported the presence in the glomerular immune deposits cases of human IMGN. Is BBAg n the circulation of normal humans, BBAb have any role in the patho- of IMGN? The answers to these s are not known, and the pathogen- MGN is open to conjecture. This escribes the detection of BBAb in uman serum.

als and Methods. Three hundred t normal adult human sera (age ears) obtained from blood donors laboratory personnel were examined. The six laboratory personnel were healthy and had normal urinalyses. Fresh, normal, Sprague-Dawley (SD) rat, rabbit, and guinea pig kidneys were quickly frozen in liquid nitrogen, and 2- to 4-μm sections were cut in a cryostat. These sections were used to detect BBAb in test sera by an indirect-immunofluorescence technique previously described (6). Fluorescein-isothiocyanate-labeled rabbit and horse anti-human IgG were obtained from Behring Diagnostics (American Hoechst Corporation, Route 202–206, North Somerville, N.J. 08876). The specificity of these was proven by double-diffusion gel precipitation and by immunoelectrophoresis.

Some of the positive sera were absorbed with a lyophilized human or rat renal cortical fraction, similar to the fraction F × IA described by Edgington and associates (7). The cortical fraction was prepared by a modification of Edgington's method. Rat cortical fraction was prepared from rat kidneys (SD) perfused *in vivo* with physicological saline. The capsule was stripped; renal cortex was separated from medulla, and the cortex was chopped with a razor blade almost to a thick paste-like consistency. It was gently pushed through a 60-mesh stainless steel wire cloth with the hlep of a spatula, and the cortical suspension was collected in a beaker placed in an ice bath. For each gram of cortical tissue, approximately 30–40 ml of physiological saline was required to obtain a uniform cortical suspension. It was centrifuged in cold (4°) at 200*g* for 3 min and washed three times with cold normal saline to obtain a clear supernatant. The sediment was saved for isolation of glomeruli. The supernatant from the initial centrifugation and subsequent washings were pooled and centrifuged in cold at 100,000*g* for 1 hr. The supernate and sediment (cortical fraction) were saved and lyophilized. Human cortical fraction was pre-

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ved.

pared in a similar manner from unperfused kidneys obtained at autopsy from patients dying of nonrenal causes. The protein content of the lyophilized cortical fractions was determined by Lowry's method. Expressed as fraction of the dry weight, the human cortical fraction contained 83% protein, and rat cortical fraction contained 75% protein. For absorption studies, measured quantities of test serum and cortical fraction were allowed to react at room tempera for 18 hr under continuous shaking as pr ously described (6). The biological acti of rat and human cortical fractions proven by their ability to induce Heym nephritis (HN) in SD rats.

Autopsied renal tissue from pati dying of nonrenal causes and renal bi specimens from patients with mini change disease were used as a sourc

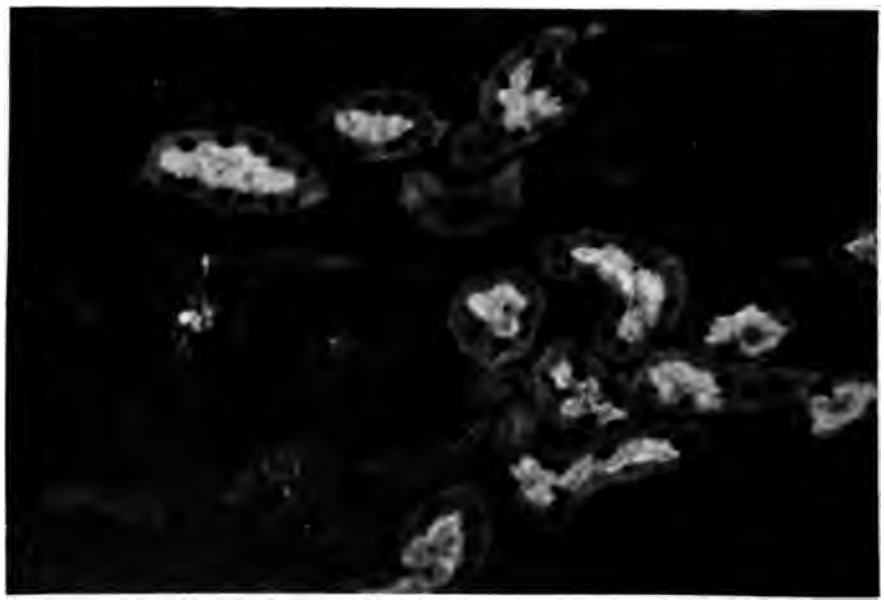

FIGURE 1

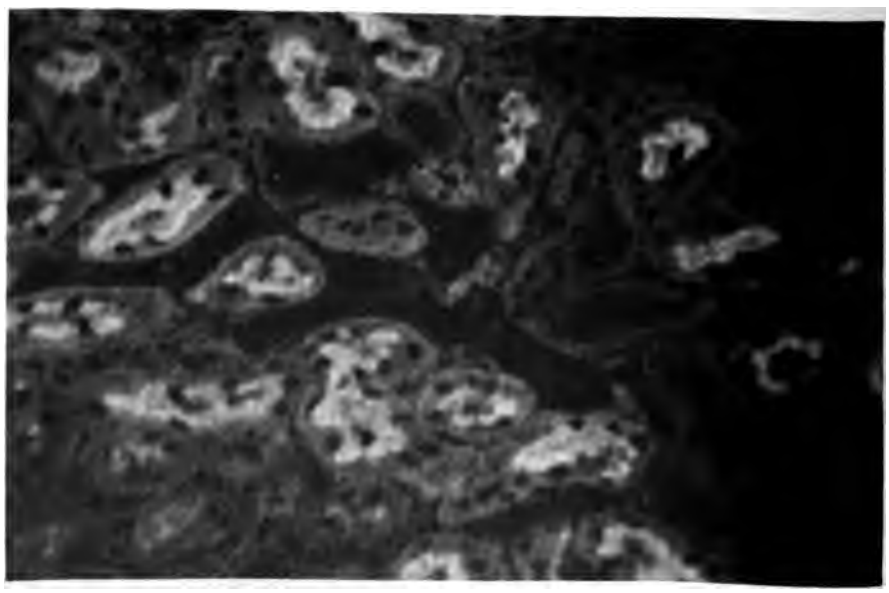

FIGURE 2

human 2- to 4-μm frozen sections. Fresh sections cut from the biopsy specimens within an hour of the biopsy were used for test sera on the same day.

Results. Thirty-six percent (128/358) of the sera examined were positive for BBAb when tested on normal SD rat kidney sections (Figs. 1 and 2). The staining was primarily localized along the brush border in the majority of the cases; however, with four sera a faint staining along the tubular basement membrane of the proximal tubule was also observed (Fig. 3). Appearance of staining along the brush border of the proximal renal tubules by normal human serum and serum from HN rats was indistinguishable. Controls using direct staining of SD rat, rabbit, guinea pig, and human kidney sections with fluorescein-labeled anti-human IgG without normal human serum were negative on every occasion. Of the 271 sera from males, 94 (34.7%) were positive, and of the 87 females, 34 (39.1%) were positive, suggesting no significant sex difference. An attempt was made to see if there was a correlation between blood groups and positive sera (Table I). Even though the sample size for blood group AB was too small, when the data for all four blood groups were analyzed by Chi-square test, it was found that persons with blood group B were significantly less prone to have BBAb in their sera ($P < 0.001$, chi-square value 15.8), and that there was no difference among blood groups O, A, and AB. To find a correlation with the Rh-factor, persons positive for BBAb were divided into Rh-positive and Rh-negative groups. Among Rh-positive subjects, 110/317 were positive for BBAb, and among Rh-negative, 13/41 were positive for BBAb. This difference was not significant; $P = 0.247$. Positive sera were also tested on human, guinea pig, and rabbit kidneys; sera showing a positive reaction on rat kidney also gave a positive reaction on guinea pig kidney, but not human or rabbit kidney. The explanation for this species difference is not apparent. Although the normal human serum did not give a positive reaction with human kidney sections, the BBAb present in the serum were partially absorbed with human renal cortical fraction. However, the rat renal cortical fraction was more effective in absorbing the

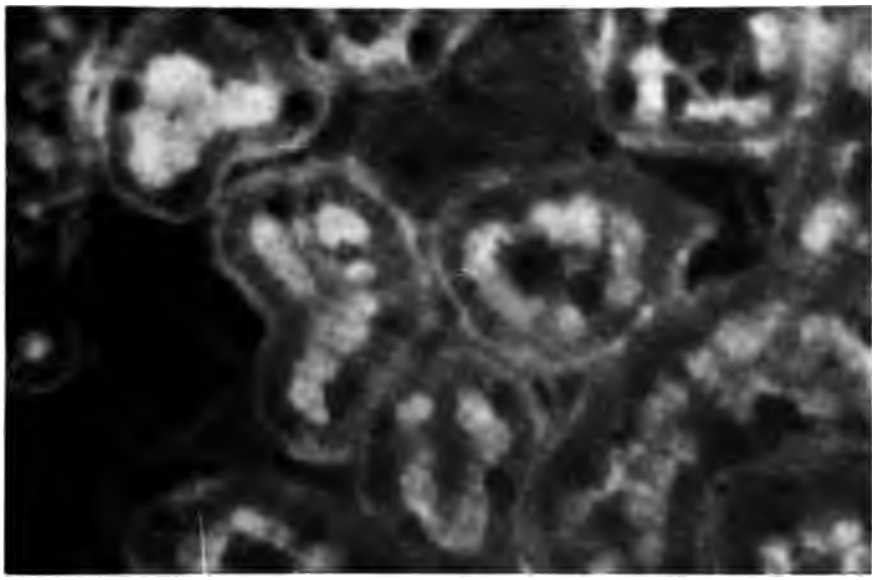

FIGURE 3

FIGS. 1, 2, AND 3. Fluorescent photomicrographs showing brush border antibodies in normal human serum by indirect immunofluorescence technique. Frozen sections from normal Sprague-Dawley rat kidney were first reacted with a normal human serum and then stained with fluorescein-isothiocyanate-labeled anti-human IgG. Bright fluorescence along the brush border region of the proximal renal tubules is seen. A part of the glomerulus seen in the right lower corner of Fig. 1 does not show any staining. Another normal human serum shows staining along the tubular basement membrane of proximal renal tubules (Fig. 3).

human BBAb (Table II). Absorption of a positive serum with other rat tissues, such as liver, spleen, and muscle, was ineffective in abolishing or reducing the positive reaction.

Sera from two patients (ages 16 and 14 years) with IMGN were also examined. Both children were found to have BBAb in their sera. The titer of BBAb in one patient was 1:8 and in the other 1:16; these titers were similar to those detected in normal human sera.

Discussion. This study has demonstrated BBAb in normal human serum reacting with rat and guinea pig kidney. However, these antibodies were not found in all human sera that were tested, and therefore, to find if only a certain group of individuals was susceptible to develop these antibodies, we attempted to see if there was a correlation between positive sera and major blood group or Rh-antigens. The Rh-factor seemed to have no correlation with the presence of BBAb; however, among the major blood groups a significantly lower incidence of BBAb was found in the B-group individuals. Why persons with blood group B have less chance of developing BBAb in their sera cannot be answered from this study. If there is any relationship between the presence or absence of BBAb in an individual's serum and the susceptibility to develop IMGN, it may prove useful to look at the blood group distribution among patients with IMGN.

Two possible explanations can be considered for the presence of these antibodies in some human sera. The first possibility is that the positive sera represent a subclinical IMGN and that 36% of the normal people are carrying a subclinical IMGN. Since urinalyses were performed on only 6/128 positive persons and renal biopsies were obtained on none, it is not possible to know if indeed these persons had subclinical IMGN. The second possibility is that the BBAb present in the normal human serum is a nonspecific heterophile antibody and is unrelated to the human IMGN. Whatever the explanation, the observation that BBAb is present in over 1/3 of normal human sera is important to keep in mind because many investigators are looking for BBAb in the sera of patients with IMGN, and if one finds BBAb in the sera or cryoprecipitates of these patients, it will not necessarily establish a causal relationship of BBAb with human IMGN.

It is of interest to note that in rats that are highly susceptible to the experimental production of HN, the author was unable to find BBAb in 1080 normal rat sera. Based on this finding it may be speculated that those humans who do not have BBAb in their sera are more susceptible to develop

TABLE I. Relationship of BBAb with Blood Groups.

	Major blood groups			
	O	A	B	AB
Positive for BBAb (%)	71 (41.3)	51 (38)	5 (11.3)[a]	1 (12.5)
Total	172	134	44	8

[a] Persons with blood group B are less likely to have BBAb in their serum; $P < 0.001$.

TABLE II. Absorption of BBAb with a Renal Cortical Fraction.[a]

		Absorption with rat cortical fraction (mg /ml)			Absorption with human cortical fraction (mg/ml)		
	BBAb titer	1	10	20	1	10	100
Human serum	1:16	Partial	Complete	Complete	None	None	Partial
Heymann nephritis rat serum	1:32	None[b]	Partial[c]	Complete[d]	None	None	Partial

[a] All absorptions were performed on undiluted sera. The degree of absorption was assessed by an estimate of the reduction in the intensity of immunofluorescence.

[b] None: no change in the intensity of fluorescence.

[c] Partial: considerable (about 50%) reduction in the intensity of fluorescence.

[d] Complete: no immunofluorescence observed (100% reduction).

and vice versa.
uggested that further studies be car-
t to understand the significance of
ıtibodies. These should include ex-
on of many more normal adult hu-
a, normal newborn human sera, sera
nts with IMGN, sera from patients
her chronic glomerulonephritides,
ıephrectomy specimens from IMGN
become available, immunoglobu-
uld be eluted from these specimens
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. It would be of interest to know if
centage of positive sera increases
:, with infants having the lowest and
:ople the highest incidence.
ıary. Brush border antibody of Hey-
ıephritis of rats was detected in
of normal human sera by indirect
ıfluorescence technique. Persons
ıod group B were less likely to de-
ıis antibody. Its existence in the se-
y or may not be related to the gene-
diopathic membranous glomerulo-
ıathy in humans. Many investigators
ing for BBAb in the sera of patients
ıal disease; thus, it is important to
ıat BBAb can exist in the sera of
ıuman controls who do not have any clinical evidence of renal disease. Further studies on this subject will be needed to understand the significance of this observation. Possibilities to explain this observation are presented.

I thank Ms. Barbara Schiffauer and Ms. Rebecca Turinic for technical assistance. This work was supported by the Kidney Foundation of Ohio, Inc., and General Research Support Grant No. RR-05410-14, NIH.

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Received June 30, 1976. P.S.E.B.M. 1977. Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 14-16 (1977)

Phosphorylation of Biologically Active Analogs of Riboflavin[1] (39592)

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Two analogs of riboflavin (Fig. 1, I), 7-ethyl-8-methyl-flavin [7-ethyl-8-methyl-10-(1'-D-ribityl)isoalloxazine] (Fig. 1, II), and 7-methyl-8-ethyl-flavin [7-methyl-8-ethyl-10-(1'-D-ribityl)isoalloxazine] (Fig. 1, III) (1), are able to serve as replacements for riboflavin in the metabolism of the rat (2, 3), with respect to growth, survival, optimal physical appearance, and efficiency of food utilization. Although these three flavins are indistinguishable by the above criteria, their utilization shows some significant differences with respect to growth (2), and especially with respect to succinic acid dehydrogenase (EC 1.3.99.1) (SDH) activity of the heart, kidney, and liver (4, 5). However, in spite of the fact that the greatest differences in the SDH activity are found in the liver, the total flavin content and the quantity of the flavin found as FAD or analog-FAD in the liver mitochondria are the same for the three flavins (6).

A third analog of riboflavin, 7,8-diethyl-flavin [7,8-diethyl-10-(1'-D-ribityl)isoalloxazine] (Fig. 1, IV) (7) is a competitive inhibitor of riboflavin in the rat (8, 9), and as reasonably expected, it mimics riboflavin deficiency particularly with respect to its influence on the SDH activity of the above tissues (5). This flavin was the first analog of riboflavin shown to be phosphorylated *in vivo* in mammalian tissue (10).

The above three analogs of riboflavin are readily isolatable and identifiable in the form of free flavins or as nucleotides in the tissue of rats to which they are administered. This was not true of the fourth analog of riboflavin to be considered, namely 7-chloro-8-methyl-flavin [7-chloro-8-methyl-10-(1'-D-ribityl)isoalloxazine] (Fig. 1, V) (11), in which case only the free flavin was found in the livers of animals receiving it (12). In terms of growth response of the riboflavin-deficient rat (13) and the utilization of food (14), this is the most active of the analogs of riboflavin.

Since these four synthetic flavins are the only analogs of riboflavin possessing significant biological activity and since phosphorylation is the first step in the conversion of these flavins into their coenzyme forms, it was of interest to study their substrate specificity for comparative purposes and for clarification of mechanisms of action.

Materials and methods. The source of the flavokinase was the livers of female rats of the Wistar strain[4] weighing about 220 g. The livers of three such animals were taken sequentially, and after each was converted to a 20% homogenate in 0.05 *M* potassium phosphate buffer, they were pooled. The enzyme preparation used was one obtained by a procedure described in the literature (15), except for the following differences. The precipitate produced by 40% ammonium sulfate concentration was discarded. The fraction obtained between 40 and 55% saturation was the most active preparation, exceeding in specific activity by a factor of 2.2, the material obtained between 55 and 75% saturation. The precipitate obtained between 40 and 55% saturation was dissolved in 0.02 *M* glycine (pH 6.9) and dialyzed as described. This enzyme preparation may be frozen to preserve it for later use; however, it lost approximately 25% of its activity after it had been frozen for 8 days (−25°).

The protein content of the flavokinase

[1] This work was supported in part by Grant No. AM 14096 from the National Institute of Arthritis and Metabolic Diseases.

[2] Author to whom inquiries concerning this report are to be sent.

[3] Some of this work was done in the Department of Biological Chemistry, University of Maryland School of Medicine.

[4] CFN rats; Carworth, Inc., New City, New York. Animals are now available from: Carworth Division, Charles River Breeding Laboratories, Wilmington, Mass. 01887.

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ı was determined by the method of *et al.* (16), and the flavokinase was described for the phosphorylation of /in. The FMN and analog-FMN de- :s were measured by the use of the ıtial extraction method of Burch *et*), as modified (18). The flavin phos- ıtion was expressed as nanomoles of ɔhosphorylated in 60 min per milli- f enzyme protein.

lts. The rate of phosphorylation of /in and the analogs of riboflavin are in Table I. The data need no restat- · elaboration. The figures in paren- following the nanomoles of flavin orylated per hour per milligram of protein express the rate of phosphorylation in the percentage of the rate for riboflavin.

Discussion. There is no apparent positive correlation between the biological activity of the flavin analogs studied and their utilization by purified rat liver flavokinase. Both the 7-ethyl-8-methyl-flavin and 7-methyl-8-ethyl-flavin are potent vitamin-like replacements for riboflavin in the rat; however, the former is phosphorylated essentially as rapidly as riboflavin (88%) while the latter is phosphorylated very slowly (8%). It is of value to note that the flavokinase activity toward the synthetic substrates is in all likelihood not a determining factor in the biological action of these analogs, so long as the enzyme does present the animal with some amount of the coenzyme. This implies that the rate of coenzyme synthesis is not limiting for the animal and that the *in vivo* coenzyme levels are not heavily dependent on the rate at which the first flavin transformation takes place.

Phosphorylated 7,8-diethyl-flavin has been observed in animal tissue before (10), and it is phosphorylated very well (69%) by flavokinase. This is particularly interesting because it argues against the presence of the ethyl group in the eight-position as the responsible factor in the low rate of phosphorylation of 7-methyl-8-ethyl-flavin. The competitive inhibitory properties of 7,8-diethyl-flavin are probably due to the competition taking place at the coenzyme level; this would help to account for this analog's potency as an antagonist.

As stated above, 7-chloro-8-methyl-flavin is active as a vitamin-like substance and as

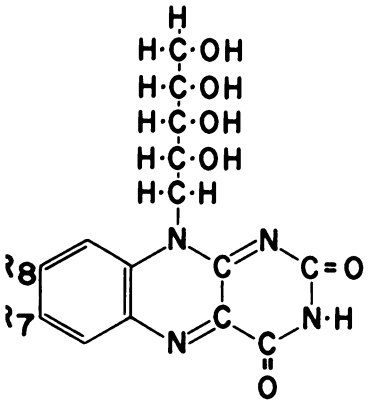

FIG. 1. Basic flavin structure.

R_7	R_8	Trivial name
H_3-	CH_3-	Riboflavin
$_2H_5$-	CH_3-	7-Ethyl-8-methyl-flavin
H_3-	C_2H_5-	7-Methyl-8-ethyl-flavin
$_2H_5$-	C_2H_5-	7,8-Diethyl-flavin
l-	CH_3-	7-Chloro-8-methyl-flavin

TABLE I. FLAVIN PHOSPHORYLATION BY PURIFIED RAT HEPATIC FLAVOKINASE.[a]

	Flavin substrate	Number of assays	Flavin phosphorylated: Nanomoles[b]	Flavin phosphorylated: Percentage[c]
I	Riboflavin	8	26 ± 1	100 ± 3
II	7-Ethyl-8-methyl-flavin	6	23 ± 2	88 ± 7
III	7-Methyl-8-ethyl-flavin	5	2 ± 0[d]	8 ± 1
IV	7,8-Diethyl-flavin	6	18 ± 1[d]	69 ± 6
V	7-Chloro-8-methyl-flavin	8	27 ± 1	104 ± 5

· different enzyme preparations using three or four pooled livers for each preparation. Some preparations ı for two different assays.

ɔmoles of flavin phosphorylated per 60 min per milligram of protein ± SE.

rate of riboflavin phosphorylation is expressed as 100% ± SE.

he following values of P (Student's t test), the symbol O! means P is 0.0001 or less. I vs II, III, IV, V = O!, 0.49; II vs III, IV, V = O!, 0.05, 0.10; III vs IV, V = O!, O!; IV vs V = O!, respectively. III and IV statistically different from all others.

an inhibitor of riboflavin in the rat. It is phosphorylated as rapidly (104%) as riboflavin. However, we have not found the coenzyme in the tissues of rats receiving this analog; we have found only traces of the free analog in such preparations. Clearly, the presence of the 7-chloro group does not influence the phosphorylation of this analog. It is interesting in this connection that 7,8-dichloroflavin is phosphorylated (15) as rapidly *in vitro* as riboflavin is, but it is devoid of biological activity in the rat and *L. casei* (19).

While it seems reasonable to expect that a flavin must be phosphorylated if it is to show activity as a vitamin-like substance or a potent inhibitor, the fact that a flavin can be phosphorylated by flavokinase in an *in vitro* system, gives us no information concerning whether it will or will not be active in the intact animal or the form of its activity.

Summary. Several analogs of riboflavin selected because of their exceptional biological activity in the rat have been studied as substrates for rat hepatic flavokinase. All of the analogs were phosphorylated, but the rate of phosphorylation showed no correlation with type of activity or potency for these activities. Phosphorylation of a flavin analog may be a prerequisite for significant biological activity but phosphorylation of a flavin does not insure that the substance will be biologically active in the whole animal.

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Received May 20, 1976. P.S.E.B.M. 1977, Vol. 154.

F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 17-21 (1977)

ıct of Hydrocortisone Acetate and Azathioprine (Imuran) on the Kinetics of ıilic Polymorphonuclear Leucocytes during an Acute Inflammation (39593)

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ıorticosteroids and azathioprine h an antiinflammatory and an im- pressive effect. This is reflected, ıple, in an altered composition of nmatory exudate. The mechanism ıg the action of these drugs is not , however.

l mechanisms have been suggested n the effect of glucocorticosteroids llular composition of inflammatory . One of these concerns an influ- the vascular wall leading to de- permeability, thus preventing the of leukocytes immigrating to the ıflammation (1-5). The effect has ı attributed to a decreased motility ıkocytes (6, 7). A third mechanism based on a lower number of circu- kocytes available for the formation flammatory exudate (8-10).

oprine is an antimetabolite with an ı DNA and RNA synthesis, thus ; cellular proliferation and protein (11, 12). The change in the com- of the cellular exudate during an ıtory response could be due to an ı of leukocyte production in the ırrow, causing a decrease in the ɔf circulating leukocytes, and thus lls are available to migrate to the e lesion (13-20).

glucocorticosteroids and azathio- ′e as a side effect a decreased host : to invading microorganisms, ıy be due to either an altered num- unction of phagocytic cells (i.e., ′tes and mononuclear phagocytes), ect on the number or function of ′tes (21, 22), or on the interaction lymphocytes and mononuclear es (23, 24). The effect of glucocor- ds (25, 27) and azathioprine (19, 20) on mononuclear phagocytes has been studied in detail, and their effect on lympho- cytes is known to some extent (21, 22), but the effect on the granulocytes during an in- flammatory response remains uncertain.

The present paper reports the effects of glucocorticosteroids and azathioprine on the number of granulocytes in the circulation and in the peritoneal cavity of mice during the normal steady state and during an acute inflammatory reaction.

Materials and methods. The materials and methods were essentially similar to those described earlier (19, 25).

Animals. In the present studies, specific pathogen-free male Swiss mice, weighing 25-30 g (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, Holland), were used.

Blood leukocyte counts. Leukocyte-counting was done in a Bürker hemocytometer; the blood was diluted 1:20 in a leukocyte pipet with Türk's solution, which contains 6% acetic acid. Differential counts were done on 200 leukocytes from blood smears fixed in absolute methanol and stained with the Giemsa stain. From these data, the number of cells was calculated for each category of leukocytes.

Peritoneal cell counts. The peritoneal cells were washed out, under strictly standard conditions, with 2 ml of phosphate-buffered saline (pH 7.2) (Difco Laboratories, Inc., Detroit, Mich.) containing heparin (50 units/ml).

Total leukocyte counts were done in undiluted, unstained cell suspensions in a Bürker hemocytometer. In the inflammation experiments, the cells were sometimes diluted 1:10 in a 0.2% trypan blue solution in saline. Differential counts were done in preparations made with a sedimentation apparatus. The cells sedimented on a microscope slide were fixed in absolute methanol and stained with Giemsa stain. These data were

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used to calculate the number of peritoneal granulocytes per milliliter of washout fluid. The peritoneal cavity of normal mice contains virtually no fluid, and the recovery of intraperitoneally injected saline is variable and always less than complete. Therefore the number of cells per milliliter is the best measure of the cellular population of the peritoneal cavity.

Drugs. Hydrocortisone acetate (kindly provided by Merck, Sharp and Dohme, Haarlem, The Netherlands) was injected sc in the nuchal region, giving a depot from which a steroid effect was sustained for at least 14 days (25). Azathioprine (Imuran, kindly provided by Burroughs Wellcome Co., London, England) was dissolved in pyrogen-free distilled water, and 0.5 ml of the appropriate dilution was injected sc in the nuchal region.

Inflammation. An acute inflammatory reaction was induced by a single injection of 1 ml of sterile newborn calf serum (NBCS, Grand Island Biological Co., Grand Island, N.Y.) into the peritoneal cavity (28, 29).

General remarks. Each animal was used only once for leukocyte and peritoneal cell sampling. Mice showing a hemorrhagic exudate were discarded. The results given for all time points represent the mean value of at least four animals.

Results. The course of the number of neutrophilic polymorphonuclear leukocytes in the peritoneal cavity and the peripheral blood during an acute inflammation. In normal animals the peritoneal cavity contains 1–2 × 10^4 polymorphonuclear leukocytes per ml, which is 0.5 to 1% of the total number of peritoneal leukocytes. From 2 hr after an ip injection of 1 ml of NBCS, the increase in the amount of fluid is no more than 0.1 ml, because NBCS is rapidly absorbed (30). During the inflammatory reaction, the number of granulocytes in the peritoneal cavity increases rapidly to a maximum of 197 × 10^4 cells/ml at 6 hr; thereafter, the granulocytes disappear rapidly (Figs. 2 and 5).

The peripheral blood of untreated mice contains 1400 granulocytes per mm^3 (range 360–4480). During an acute inflammatory response induced by NBCS, the number of peripheral blood granulocytes increases until a peak level of (4800–6000 cells/mm^3) is

140 × 10^4 cells). When the animals
›retreated with 3 mg/kg of azathio-
the response of the polymorphonu-
:ukocytes in the peritoneal cavity was
ightly (10%) lower (210 × 10^4 cells/
12 hr) than that in the untreated ani-
Fig. 5).
r treatment for 4 days with 200 mg/
ızathioprine, the number of granulo-
n the circulation decreases to 660/
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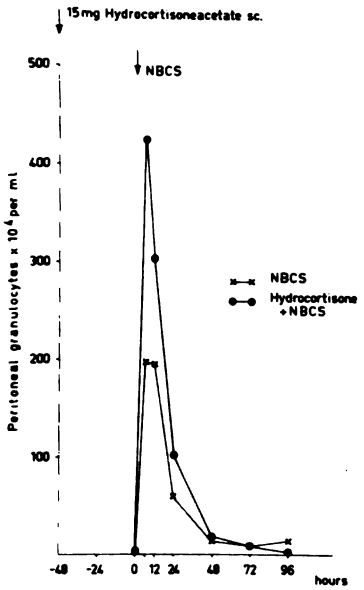


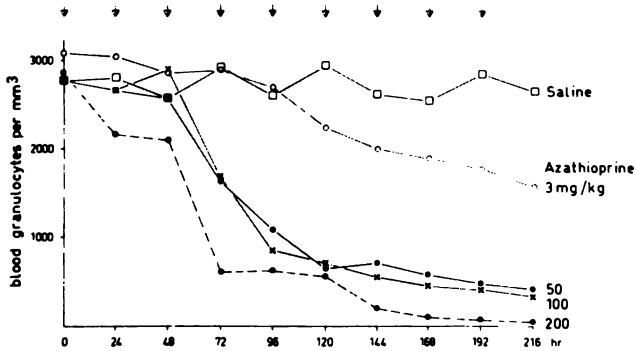
2. Course of the number of granulocytes in
toneal cavity during an acute inflammatory re-
ıduced by an ip injection of newborn calf serum
treated with hydrocortisone acetate and in
mice.

number of neutrophilic polymorphonuclear leukocytes to 3535/mm^3 at 6 hr, but thereafter the number of granulocytes drops rapidly (Fig. 4). Animals treated with a lower dose of azathioprine (3 mg/kg) showed no decrease in the number of peripheral blood granulocytes. When these animals received an inflammatory stimulus, the granulocytes increase to a peak value of 5660 granulocytes/mm^3 at 6 hr. Initially, this response is similar to that in normal mice, but at later time points, the number of granulocytes is lower in treated than in untreated animals (Fig. 4).

Discussion. The main conclusion to be drawn from this study is that neither hydrocortisone acetate nor azathioprine causes a significant reduction in the number of neutrophilic granulocytes in the exudate of an acute inflammatory lesion.

Hydrocortisone increases the number of circulating granulocytes, as found earlier (3), which implies that granulocytes are available to migrate into the site inflammation. The course of the peripheral blood neutrophils during the acute inflammatory response indicates that new cells are also recruited from the bone marrow into the peripheral blood. Since the number and rate of appearance of granulocytes at the site of the lesion is not decreased by hydrocortisone, it is unlikely that vascular permeability for the granulocytes or the motility of these cells is affected either.

The effect of hydrocortisone on the neutrophils differs widely from that on the mononuclear phagocytes, since this drug causes a severe monocytopenia in the



3. The effect of treatment with various doses of azathioprine on the number of peripheral blood
cytes.

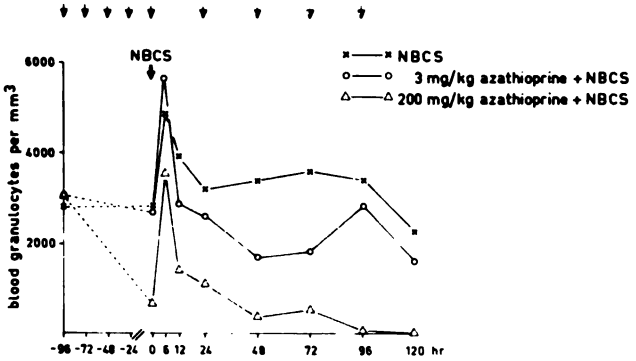


FIG. 4. Course of the number of peripheral blood granulocytes during an acute inflammatory reaction in the peritoneal cavity under treatment with azathioprine and in control mice.

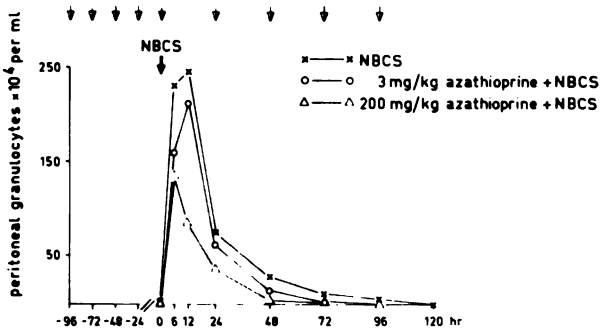


FIG. 5. Course of the number of granulocytes in the peritoneal cavity during an acute inflammatory reaction caused by an ip injection of newborn calf serum in mice treated with azathioprine and in control mice.

mouse, the rat, the guinea pig, the rabbit, and man (25, 32–35); and the absence of an increase in the number of mononuclear phagocytes in the inflammatory exudate is explained by the severe monocytopenia that cannot be overcome by the inflammatory stimulus (25).

The action of azathioprine is quite different. This drug inhibits the proliferation of the granulocytes' precursors in the bone marrow, thus bringing about a decrease in the circulating granulocytes after 4 days of treatment with a high dose of the drug. However, the stimulus of an acute inflammation induces the release of a sufficient number of (mature) granulocytes from the bone marrow pool into the peripheral blood, which then migrate to the site of the lesion. Treatment with a low dose, which is about equivalent to a nontoxic, immunosuppressive, antiinflammatory dose in man (19), does not affect the circulating granulocyte level, however, and does not impair the granulocyte response during an acute inflammation.

It is of interest that the effect of azathioprine on the neutrophilic polymorphonuclear leukocytes differs from that on the mononuclear phagocytes. A low dose of this drug causes a reduction in the number of circulating monocytes, and the influx of mononuclear phagocytes into the site of inflammation is significantly shorter and smaller than in the absence of azathioprine (19, 20).

It may be that the short duration of the neutrophil response during an acute inflammation masks a possible effect of hydrocortisone or azathioprine, since for such a short response enough granulocytes are still available in the bone marrow reserve pool. However, if there is a sustained need for granulocytes during treatment with these drugs, too few cells might be available and the granulocytes' response will be abnormal.

Summary. The course of the number of

ilic polymorphonuclear leukocytes
. acute inflammation in the perito-
y was studied in mice treated with
tisone or azathioprine. The results
t in these animals the number of
ilic granulocytes appearing in the
tory exudate is similar to that in
ıot treated with these antiinflam-
rugs. This study indicates that glu-
steroids do not affect the vascular
lity for granulocytes or the motility
:ells, and that during azathioprine
t the bone marrow contains a suffi-
ıber of neutrophils that can be re-
:o the circulation.

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Received January 12, 1976. P.S.E.B.M. 1977, Vol. 159.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 22-26 (1977)

Bile Acid Composition of Some Lizards from Southwestern United States[1] (39594)

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Haselwood suggested a relationship between the chemical types of bile acid and the systematic classification of vertebrates (1). Our knowledge of bile acid composition in different vertebrates is limited to only a few species and families, and more comparative data are needed at species, generic, and family levels. Only after the analysis of such data can realistic and worthwhile generalizations be developed concerning possible evolutionary patterns for bile acids in vertebrates.

Lizards are both a phylogenetically diverse and an ancient group which is found throughout much of the world in a wide array of ecologic roles and habitats (2). Studies of bile acid composition in this group would greatly aid in our understanding of the relationship of chemical structure of bile acids and their evolution within vertebrates. As an initial stage in such an investigation we have identified the bile acids from nine species of lizards all common in southwestern United States. Two lizard families, Gekkonidae and Teiidae, are represented by a single species, while the ecologically diverse Iguanidae is represented by seven species. This allows for comparisons of bile acid composition between three families, between genera within a family, between species of the same genus, and between species which differ in diet and habitat.

Materials and methods. Bile was obtained from the gallbladders of 31 lizards of nine different species from southern Nevada. Lizards were collected alive and sacrificed within 3 days of capture. The gallbladders were removed intact and frozen until analysis.

The bile (0.2–1.0 ml) was extracted by the addition of 10 ml of hot ethanol–methanol and shaking (3). The precipitated protein was removed by centrifugation at 2000*g* at 4° for 15 min. The alcoholic extract was decanted, and the precipitate was washed with 2 ml of hot ethanol–methanol and filtered. The extracts were combined, were diluted to 40% alcohol with distilled water, and were delipidated with petroleum ether. The aqueous alcohol solution was then evaporated to dryness under nitrogen *in vacuo*. The residue was dissolved in 1 ml of methanol.

An aliquot of the bile acid extract was applied as a spot to a tlc plate (20 × 20 cm) coated with a silica gel G (Merck & Co.) in a 0.25-mm thick layer. The plates were developed in *n*-butanol–glacial acetic acid–water (10:1:1, v/v for 4 hr) (4). After evaporation of the solvents, the bile acids were located by iodine vapor, and relative R_f values of any bands were compared to known standards of free cholic acid (CA), chenodeoxycholic acid (CDCA), and their taurine and glycine conjugate for identification. The rest of the bile extract was hydrolyzed at room temperature (24 hr) with acetone–ethanol (9:1) adjusted to pH 1.0 with 2 *N* HCl (5). The solvents were then evaporated and subjected to alkaline hydrolysis as previously described, and free bile acids were extracted (3). Bile acids were measured by gas–liquid chromatography (glc) and for this purpose the bile acids were converted into methyl esters by methanol 2,2-dimethoxy-propane (Aldrich Chemical Co., Milwaukee, Wisc.) and concentrated HCl (6). Prior to glc, the

[1] Supported in part by NSF Grant No. GB 35281 and MRC Grant No. MA 4865.

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esters were converted into trifluo-
e by reaction with 0.3 ml of trifluo-
anhydride at 40° for 40 min. The
yses were performed on a Packard
is chromatograph system equipped
al glass columns (1.2-m × 2-mm
ntaining 3% OV-210 on 80–100
s Chromosorb W (Chromatographic
ies, Ontario). The separation was
225° isothermally using helium (30
as the carrier gas. The glc system
brated by means of standard mixture
sly described (3). The known bile
ethyl ester were quantitated using
anic acid as internal standard (3).
samples were further analyzed on
:d gas–liquid chromatography–mass
netry (glc-ms) Varian Mat CH-5 sin-
sing mass spectrometer coupled to a
Mat computer as previously de-
(6).
icid percentages were compared be-
amilies and between the seven ig-
pecies using a similarity index (IS)
:d previously (7):

$$IS = \frac{2M_w}{MA + MB} \times 100,$$

M_w refers to the sum of the smaller
itive values common to two samples,
he sum of the quantitative values for
iple, and MB is the sum of the values
other sample. Since both samples
ve a total quantitative value of 100,
nula can be rewritten as:

$$IS = \Sigma M_w.$$

ts. The tlc of the conjugated bile
the nine species studied showed only
ile acids, and no traces of sulfate,
conjugated bile acids, or free bile
ould be detected. The glc of the spe-
lied is shown in Fig. 1. It is apparent
st of the species studied contained at
'e peaks which were further identi-
glc-ms as allocholic acid (AC),
acid (CA), chenodeoxycholic acid
.), deoxycholic acid (DOCA), and
olic acid (LCA). The molar percent-
:ach bile acid varied from species to

iverage molar percentage of differ-
acids for each species is shown in Table I. In Gakkonidae and Teiidae, CA was 70 and 44%, respectively, as compared to 2–13% in the seven species of Iguanidae. The seven species of iguanids studied all exhibited high percentages of AC (64–91%) as compared to 6% in the other two families. Only trace amounts of LCA were detected in all species except *Cnemidophorus*. In *Cnemidophorus* an additional bile acid, representing 6% of the total, was not identified.

The bile acid spectra for the three lizard families are further analyzed in Table II, where IS values for bile acid percentages shared between families were calculated (7). These indices provide a method for determining the degree of similarity in the total pattern of bile acids for different groups. Bile acid spectra of Gekkonidae and Teiidae showed a high IS as compared with Iguanidae. Similarly, bile acid spectra of the seven species of iguanids are compared in Table III. Similarity indices were consistently high ranging from 73 to 97%.

Discussion. Lizard bile acids have been reported for only a few species. Haslewood (1) in two species of *Anolis* (Iguanidae) reported the presence of AC and CA, but recently Okuda *et al.* (10) studied the bile acid of *Iguana iguana* (Iguanidae) and reported the presence of AC as the major bile acid and 3α-, 7α-, 12α-trihydroxy-5α-cholestan-26-oic acid as the second major bile acid in this species, but CA was not detected. Their finding was supported by the lack of enzymatic activity to reduce 7α-hydroxycholest-4-en-3one to 5β alcohol in the liver as reported by others (11).

In our study, AC and CA were found in all species studied (Table I). Among the iguanids studied, AC was the primary bile acid which is compatible with findings for *Anolis* and for *Iguana iguana* (1, 10). Evidently, the molar percentages of AC and CA are variable among iguanids and should be studied further, particularly for more species. Of considerable importance is the low concentration of AC in the single species studied in both the Teiidae and Gekkonidae. Also, CDCA, DOCA, and LCA were found in varying amounts in all but one of the species studied.

Reptiles are an ancient and largely extinct

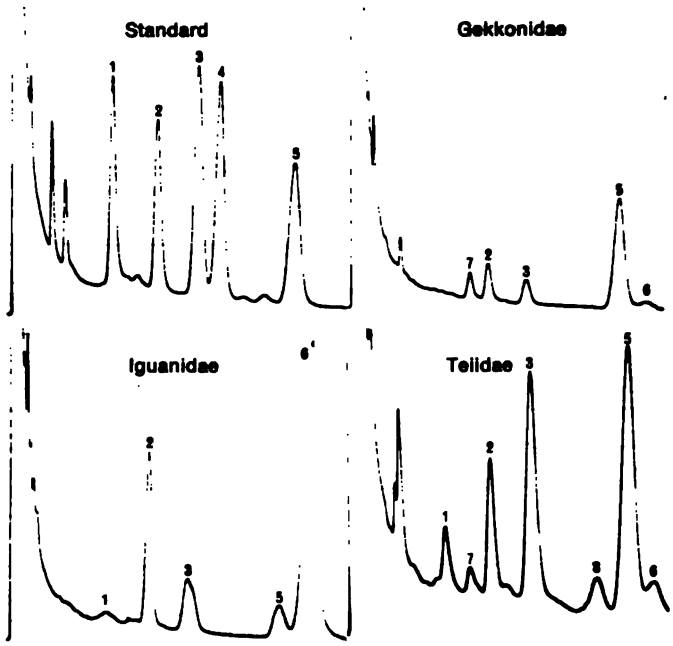

FIG. 1. A typical gas chromatogram of trifluoroacetates of bile acid methyl esters. Column 3% OV-210 80–100 mesh Chromosorb W operating temperatures were: Column 225°, injection 225°, and detection 240°. T carrier gas was helium and its flow was 37.5 cc/min. Chart speed 5 min/2.5 cm. The numbers 1 to 8 represent following: 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxycholic acid; 4, hyodeoxycholic acid; 5, ch acid; 6, allocholic acid; 7, unknown; 8, unknown.

TABLE I. CLASSIFICATION, SAMPLE SIZE, BODY WEIGHT, AND MOLAR PERCENTAGE OF DIFFERENT BILE ACIDS FROM BILE OF LIZARDS.

Classification and common name	Sample size	Av body wt (g)	Allocholic acid (AC)	Cholic acid (CA)	Chenodeoxycholic acid (CDCA)	Deoxycholic acid (DOCA)	Lithocholic acid (LCA)	Oth
Infraorder Gekkota								
Family Gekkonidae								
Coleonyx variegatus (Western banded gecko)	4	2.2	5.7	69.5	10.5	14.2	T[b]	T[b]
Infraorder Iguania								
Family Iguanidae								
Dipsosaurus dorsalis (Desert crested lizard)	7	44.0	85.0	2.1	–[a]	12.9	T[b]	–[a]
Sceloporus magister (Desert spiny lizard)	3	40.0	63.7	6.7	7.3	22.3	T[b]	–[a]
Sceloporus occidentalis (Western fence lizard)	7	16.0	85.9	10.1	1.3	2.8	T[b]	–[a]
Sceloporus graciosus (Sagebrush lizard)	1	6.0	84.6	8.7	2.1	4.6	T[b]	–[a]
Urosaurus graciosus (Long-tailed brush lizard)	1	4.0	90.0	5.0	5.0	T[b]	T[b]	–[a]
Uta stansburiana (Side-blotched lizard)	2	3.0	75.9	12.9	6.1	5.1	T[b]	–[a]
Phrynosoma platyrhinos (Desert horned lizard)	5	24.0	91.0	1.6	1.0	6.4	T[b]	–[a]
Infraorder Scincomorpha								
Family Teiidae								
Cnemidophorus tigris (Western whiptail)	2	11.5	6.0	44.4	25.8	14.2	3 5	6.0

[a] –, Is not detectable.
[b] Trace is less than 0.1%.

snakes and lizards making up the recent reptile fauna (2). Lizards, are almost worldwide in distribu- he exception of arctic and anarc- . The three families studied are related phylogenetically, being three separate infraorders. The ie are primarily an old world i related families of Jurassic ori- species are present on all conti- pt Antarctica. Fossils from fami- to the Iguanidae are also of Jur- i. This family, with the exception sil genera, is largely restricted to orld. The Teiidae is a group of lizards which date back to Creta- . Therefore, the three lizard fami- l all evolved during the Mesozoic Gekkonidae of old world origin, Iguanidae and Teiidae of either origin or major affinity.

prising that the IS between Gek- id Teiidae is so high (75%), con- at they are distantly related phy- ly and had their major evolution new world, respectively. In con- guanidae have extremely low IS en compared with either group. n on possible evolutionary rela- ased upon bile acid compositions curtailed, however, until further her species of teiids and gekkon- ducted.

y indices for the seven species of e surprisingly high. In general, IS species of rodents belonging to the same family are considerably lower (12). Within the three species of *Sceloporus* studied, IS values were lower in one instance than most comparisons between genera. In part, the high IS values are due to the high percentages of AC found in all species which allow for only minor differences in the percentages of the other bile acids.

The portion of various bile acids appear to be little affected by diet as the single vegetarian, *Dipsosaurus dorsalis*, has comparable IS values with the insectivorous species. The nine species studied occur in a wide variety of habitats in the Mohave Desert (13). There appears to be no apparent relationship between ecologic distribution and IS values.

To our knowledge this is the first study concerning the quantitative distribution of bile acids in a large group of lizard species occurring in the same geographic region. It represents a start in our understanding of bile acid relationships among lizards. We plan further studies involving more families, genera, and species, which will hopefully allow meaningful analysis of evolutionary and biochemical relationships.

Summary. Nine species of lizards representing three families were used to investigate bile acid composition of the bile acid pool isolated from gallbladders. Members of the three families contained AC, CA, CDCA, DOCA, and LCA in varying proportions. Gakkonids and teiids, represented by a single species each, had quite similar bile acid spectra, with CA as the primary bile acid. This is surprising since these families have different geographic origins and are only distantly phylogenetically related. In contrast, the seven species of iguanids had AC as the primary bile acid. At both generic and species levels, the iguanids studied had quite similar bile acid spectra.

I. BILE ACID SIMILARITY INDICES FOR LIZARD FAMILIES

	Teiidae	Iguanidae[a]
nidae	75	23
e		24

percentages for the seven iguanid species d.

TABLE III. BILE ACID SIMILARITY INDICES FOR SEVEN SPECIES OF IGUANID LIZARDS.

	Sceloporus occidentalis	*Sceloporus graciosus*	*Uta stansburiana*	*Dipsosaurus dorsalis*	*Urosaurus graciosus*	*Phrynosoma platyrhinos*
agister	75	77	82	79	74	73
:cidentalis		97	90	90	92	91
'aciosus			91	91	92	92
ana				83	86	84
dorsalis					87	93
aciosus						93

Within this group, food habits or ecologic distribution appeared to have little effect on the proportion of bile acids.

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Received October 22, 1974. P.S.E.B.M. 197 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 27-29 (1977)

icide Effects on the Immune Response and Metabolic Activity of Chicken Lymphocytes[1] (39595)

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use of DDT (1,1,bis-[*p*-chloro-2,2,2-trichloroethane) and mirex ichlorooctahydro-1,3,3,-methenoobuta[*cd*]pentaline) have caused rable discussion in recent years be- f their potential effects on biological . As a result of this, prodigious s of research have been generated to arious aspects of physiological sys- 'et a paucity of information exists on :cts of pesticides on the immune re- and relatively few publications have :d over the years (1-5).

(3) administered DDT and mirex he neonatal period in order to deter- ieir potential for disrupting bursal ment and future antibody produc- ice chemical interference with bursa ieonatal development (6-11) will re- :limination or reduction in immuno- l G, antibody production, and matu- of plasma cells. Glick's (3) findings that these pesticides were not capa- iitiating bursal involution during the eeks of life. However, DDT treat- d cause a reduction in immunoglob- and M. This interesting observation explainable on the basis of bursal e and directed us to other avenues of

: present study, metabolic activity of cytes from different lymphoid com- its and the immune response of :reated with DDT and mirex were :d. The splenic and thymic lympho- :monstrated a marked reduction in :tabolic activity upon pesticide treat- 'his observation is further supported atio of 7 and 19 S antibody levels.

ials and methods. Pesticide treat- Technical grade mirex (M) and 99.0+% pure *p,p*-DDT (D) were obtained from Allied Chemical and Aldrich Chemical Companies, respectively. Weighed samples of each pesticide were added to soybean oil premix and then combined with basal feed (12) to yield 100 ppm (M-100 or D-100) level. The chickens were from a strain of New Hampshire, an American breed, developed by Professor L. J. Dreesen of our Poultry Department. All eggs were hatched in Jamesway incubators. Feed and water were supplied *ad libitum* from the day of hatch.

Immunological data. Sheep red-blood cells (SRBC) stored in Alsever's solution was used as the antigen. The cells were washed 3 times in physiological saline (0.9% NaCl) prior to use. Twelve 4-week-old birds from each of the three experimental groups were immunized intravenously (iv) with 1 ml of a 5% suspension. A microtiter procedure was followed for antibody titrations (13, 14). The birds were bled from the brachial vein on the fourth, seventh, and tenth day after the antigen injection to collect serum for the micromethod. Serum samples were titrated individually (15). Titers were expressed as the logs of the reciprocal of the highest dilution giving visible agglutination.

Equal volumes of serum and 0.2 *M* mercaptoethanol (ME) in phosphate-buffered saline (pH 7.4) were mixed and incubated at 37° for 30 min prior to serial dilution (16). Agglutination tests were conducted and the titer was recorded as ME-resistant antibody.

Metabolic activity measurement. The method of preparation of thymus, bursal, and splenic lymphocytes was similar to the one used previously in our laboratory (17, 18). The bursa, spleen, and thymus from each bird were rinsed individually in Hank's balanced salt solution (HBSS) and cut into small fragments. They were aspirated

ial article Number 3302 from the Mississippi ral and Forestry Experiment Station.

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ierved.

through a disposable-needleless syringe (5 ml) and were filtered through a lens paper. The cells were washed twice with Ringer's solution and resuspended in Ringer's solution after the final washing. The cell counts were volumetrically adjusted to 25 × 10^6 cells/ml. A cell viability count was also performed for each cell suspension using eosin Y exclusion technique (19). There were 10 birds per treatment.

Oxygen uptake studies were conducted with a YSI Model 53 biological oxygen monitor at 37°. The test samples contained 2 ml of cell suspension (25 × 10^6 cells/ml), 0.3 ml of fresh chicken serum from the same bird, and 0.1 ml of glucose (30 *M*). The Ringer's solution was saturated with air at 37° and contained 4.95 μl O_2/ml of solution. Oxygen consumption was determined by calculating the loss of oxygen from the solution during a 15-min period.

Statistical analysis. The agglutinin titers and oxygen consumption rates were analyzed by the analysis of variance (20). Significant mean differences were determined by Duncan's new multiple range test (21).

Results. Feeding mirex and DDT from hatch to 40 days of age did not influence weights of body, bursa, spleen, thymus, or liver during the entire period of the experiment. There were no significant differences in total antibody production in normal and pesticide fed birds when measured by agglutinin titers (Fig. 1). However, DDT and mirex treatment significantly suppressed the ME-resistant antibody (IgG) and markedly increased ME-sensitive antibody (IgM) production at 5 and 7 days postantigen injection (Fig. 1).

The oxygen consumption for thymic and splenic cells was significantly lowered in the presence of both mirex and DDT (Table I). On the other hand, oxygen consumption of bursal cells was not consistently influenced by the pesticides.

Discussion. The agglutinin titers to a primary response of SRBC were normal in pesticide-treated birds. However, the ME-resistant (IgG) antibody was significantly lower and the ME-susceptible (IgM) antibody significantly higher in the pesticide-fed birds. These perturbations in the class of antibody of pesticide-treated birds cannot be attributed to a toxic effect of the pesticide on the bird since body weight was normal, nor can they be attributed to an overt catabolic influence on central peripheral lymphoid tissue since the weight of the bursa, thymus, and spleen in the DDT and mirex birds did not differ from the control birds.

The subtle influence of the pesticides on T-cells was reflected in the reduced oxygen consumption of thymic lymphocytes subsequent to DDT and mirex feeding. Failure to obtain a consistent change in O_2 uptake by bursal lymphocytes would suggest that the significant reduction in O_2 uptake of splenic cells from pesticide-fed birds was a reflection of a reduced T-cell function.

The reduced metabolism of T-cells would, in part, account for the depressed IgG antibody titers of DDT and mirex birds since this class of antibody is known to be dependent of T-cell function (22–24), and interaction of T- and B-cells is a prerequisite for normal antibody response to a variety of antigens (22, 25–27). The similarity in total

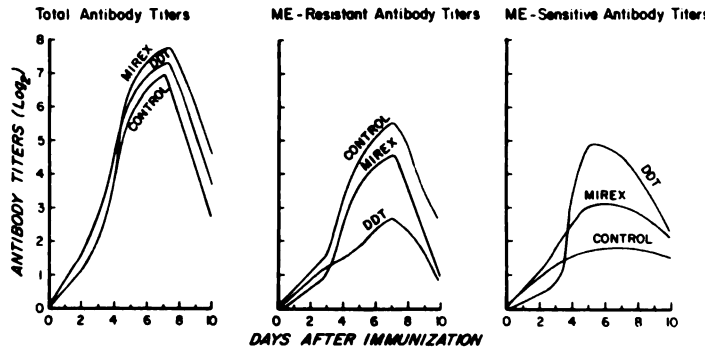

FIG. 1. Effect of pesticides on the antibody response to sheep red-blood cells.

ABLE I. OXYGEN CONSUMPTION (MICROLITERS PER HOUR) OF THYMUS, BURSAL, AND SPLENIC LYMPHOCYTES FROM CONTROL CHICKENS AND DDT- AND MIREX-FED CHICKENS.[a]

atment	Thymus	Bursa	Spleen
Weeks			
Control	9.75 ± 0.33[b]	8.48 ± 0.35[b]	14.43 ± 0.48[b]
Mirex	6.28 ± 0.38[c]	7.59 ± 0.43[b,c]	10.81 ± 0.41[c]
DDT	5.64 ± 0.29[c]	6.87 ± 0.23[c]	8.44 ± 0.61[d]
Weeks			
Control	12.74 ± 0.92[b]	9.02 ± 0.85[c]	14.89 ± 0.79[b]
Mirex	9.12 ± 0.73[c]	11.35 ± 0.81[b]	12.74 ± 0.92[c]
DDT	8.23 ± 0.82[c]	10.14 ± 0.51[b,c]	8.83 ± 0.82[d]

s and standard errors for 10 observations. Means not possessing the same superscript within each e significantly different at 5% level (21).

in titers for the three groups sug- normally functioning B-cell. Further : that the B-cell in pesticide-treated normal could be obtained by utiliz- ymic-independent antigen.

ary. Immune response and meta- tivity of bursal, splenic, and thymic ytes from chickens treated with d Mirex were evaluated. The results d a marked reduction in the meta- ctivity upon pesticide treatment. as no significant difference in total / production, but IgG levels were ntly reduced and IgM levels were y elevated. The significance of re- etabolic activity and IgG produc- e discussed in relation to T-cell ac-

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Received May 12, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 30-32 (1977)

Failure of Sodium Iodide Loading To Inhibit Renin in the Rat (39596)

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Inhibition of renin secretion by NaCl administration has been attributed to detection of a signal in renal baroreceptors or at the macula densa (1). KCl (2), HCl (3), and $CaCl_2$ (4) inhibit renin release, and active Cl transport has been demonstrated in the ascending limb of Henle's loop (5). These observations among others have led to the hypothesis that chloride may be a principal ion signaling alterations in renin release at the macula densa (6). We have demonstrated that $NaHCO_3$ and $KHCO_3$ loading fail to inhibit renin in the rat, supporting the hypothesis that the signal produced at the macula densa may be modified substantially by the anion delivered with Na or K (7). We have extended these studies by comparing the renin response in the rat to chronic loading with equimolar amounts of NaI or NaCl.

Methods. Twenty-four adult male Sprague–Dawley rats were given free access to deionized water and a low NaCl feed (International Clinical and Nuclear Corp., Cleveland, Ohio) for 1 week. This feed contains 274 μEq of K^+, 1 μEq of Na^+, and 2 μEq of halide per g determined following nitric acid digestion in our laboratory. The animals were then divided into three groups of eight rats each and were placed in individual metabolic cages for the second week of the study. For drink, group A was given NaI, 75 mEq/liter; group B, NaCl 75 mEq/liter; and group C, deionized water. All animals continued on the low NaCl diet for the duration of the study. To assure a similar intake of feed and drink, group A rats were studied 1 day in advance of groups B and C, which were maintained according to a paired feeding schedule. Daily sodium, potassium, halide, and water balances were determined for each animal, based on measurement of dietary intake and urinary excretion.

After 7 days on this regimen, all animals were sacrificed by decapitation, and blood emanating from the trunk was collected in chilled EDTA-containing tubes for subsequent determinations of plasma renin activity (PRA) and urea nitrogen concentration. A single kidney was harvested from each animal for subsequent determination of renal renin content (RRC).

Na and K concentrations in feed and urine were measured by flame photometry and urea nitrogen by the method of Crocker (8). Total halide (Cl and I) was measured with a Buchler chloridimeter (Buchler Instruments, Ft. Lee, N.J.).

PRA was measured in quadruplicate with the radioimmunoassay procedure of Haber (9). Renal renin was extracted and quantified as previously described (10). One unit of renal renin is arbitrarily defined as that concentration of renin that will generate 100 ng of angiotensin I during a 15-min incubation in the presence of excess sheep renin substrate.

In instances where data were compared for two groups, statistical significance was determined with Student's t test. When data were compared among three groups and when the variance for the three groups was similar, the significance of group comparisons was computed with analysis of variances. Because analysis of variance requires similar group variances, for several three-group comparisons with dissimilar variances statistical significance was computed with the Wilcoxon rank sign test (11). Significance is at the 0.05 level.

Results. Initial and final body weights among the three groups did not differ (Table I). Plasma urea nitrogen concentration in group B was significantly different from ($P < 0.05$) that in group A, but neither was different from control.

Net fluid balance in groups A, B, and C did not differ (Table II). Net sodium balance was not significantly more positive ($P > 0.1$) in NaI-drinking animals than in those drinking NaCl and was greater than controls in both electrolyte-drinking groups. Net po-

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balance among the three groups did
ter ($P > 0.7$). Total halide balance
t different between the electrolyte-
g rats but was significantly greater (P
) in both groups A and B than that
up C. Although the method for de-
ng halide does not distinguish be-
chloride and iodide, it is likely that
f the measured halide excretion in
A represents iodide and in group B
e.

. (4.0 ± 1.2 ng/ml/hr) in NaCl-drink-
s was suppressed significantly ($P <$
when compared to NaCl-deprived
s (7.9 ± 1.8 ng/ml/hr) (Fig. 1). How-
NaI-drinking rats, PRA (9.8 ± 0.8)
t different from ($P > 0.1$) control
RRC changed in the direction of the
ons in PRA but the magnitude of the
was not statistically significant ($P >$

ussion. Unlike dietary loading with
NaI loading failed to suppress PRA.
e there were no significant differ-
n initial and final body weights and
nd sodium balance in NaI-drinking
mpared to NaCl-drinking rats, it

I. BODY WEIGHTS AND RENAL FUNCTION.[a]

p	Initial body weight (g)	Final body weight (g)	Plasma urea nitrogen (mg/dl)
3)	313 ± 7	294 ± 9	27 ± 1*
)	311 ± 7	303 ± 8	20 ± 2
3)			
r)	302 ± 6	291 ± 9	23 ± 1
3)			

ues given are mean ± SE; n is indicated in
ses. No statistical difference (P-NS) between
g groups unless indicated. * $P < 0.05$ com-
group B.

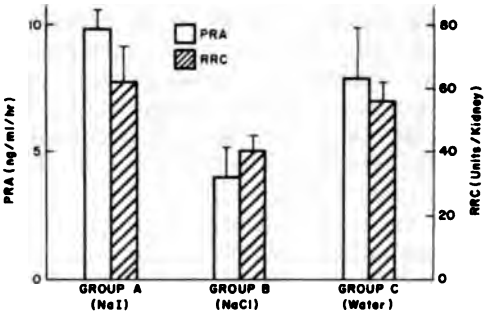

FIG. 1. PRA and RRC in groups A, B, and C.

seems unlikely that volume contraction in the former group can explain the failure of renin inhibition. Potassium balance was similar among the groups suggesting that failure of NaI to suppress renin is not related to an effect on potassium.

RRC was not suppressed significantly by 7 days of loading with 0.075 *M* NaCl although, similar to PRA, RRC tended to be decreased in NaCl- but not in NaI-drinking animals. We have demonstrated previously that RRC in the rat is suppressed after 1 week of drinking 0.15 *M* NaCl (7). The present results suggest that, although PRA is suppressed, 0.075 *M* NaCl for 1 week in rats on a low sodium diet is not a sufficiently potent stimulus to inhibit RRC.

In the rat, active chloride transport has been demonstrated in the ascending limb of the loop of Henle (5), and the signal perceived by the macula densa during saline loading may be NaCl transport rather than Na load (12). Iodide is reabsorbed passively and coextensively with chloride in the nephron (13), and micropuncture studies have shown a lower permeability for iodide than for chloride in both the proximal and distal tubule of the rat (14, 15). However, in the presence of low chloride excretion

TABLE II. NET FLUID AND ELECTROLYTE BALANCE.[a]

Group	Fluid (ml/7 days)	Sodium (μEq/7 days)	Potassium (μEq/7 days)	Halide (μEq/7 days)
(NaI) (n = 8)	45 ± 3	3062 ± 238*	3489 ± 895	1914 ± 162*
(NaCl) (n = 8)	52 ± 6	2336 ± 306*	3782 ± 646	2119 ± 302*
(Water) (n = 8)	46 ± 7	−68 ± 45	3984 ± 836	33 ± 29

ues given are mean ± SE; n is indicated in parentheses. No statistical difference (P-NS) between or roups unless indicated. * $P < 0.01$ compared to group C.

rates (a condition likely to be present in group A and group C animals of the present studies), Walser and Rahill provided evidence for active iodide transport (13). The failure of NaI to inhibit renin is consistent with the hypothesis that Na-induced inhibition of renin is mediated by NaCl transport across the macula densa. The demonstration that iodide cannot substitute for chloride in this role suggests that the active transport process for chloride at the macula densa is highly specific.

Summary. NaCl-deprived rats given NaI to drink failed to suppress PRA compared to rats drinking equimolar amounts of NaCl, despite a more positive Na balance in the former group. Volume contraction and potassium deficiency do not appear to explain this observation. If chloride is a principal ion to signal the macula densa to release renin, our data suggest that iodide cannot substitute in such a role.

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: of Flow Rate and Glucose Concentration on Glucose Uptake Rate by the Rat Limb (39597)

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etal muscle is responsible for about f the resting oxygen consumption (1), us the rate of nutrient uptake by this :an greatly influence the pool of nutri- vailable to other tissue. Free fatty re the great source of fuel, but resting l muscle also consumes glucose. only 10–37% of the oxygen consump- resting skeletal muscle has been at- d to glucose oxidation (2, 3), the im- ce of muscle as a "glucose sink" can lized in diabetes mellitus when the : uptake rate of skeletal muscle is lly reduced.

vo a number of factors can influence e of glucose uptake by skeletal mus- ıe permeability of muscle to glucose e effect of insulin have undoubtedly d the most attention. No studies ɔe located in which the effect of flow ı the glucose uptake rate by skeletal was studied, although this parameter :n investigated in the brain (4, 5).

owing number of metabolic studies perfused muscle preparations in low rate to the muscle is set to main- : desired arterial pressure (6–9). Of- :se flow rates are much above the ogical range for resting muscle blood What effect, if any, this has on the : uptake rate of muscle has not been ined. The purpose of the present in- tion is to determine the influence of : concentration and flow rate on the glucose uptake by the perfused rat ıb (basically a muscle preparation).

rials and methods. Surgical prepara- lale rats (Charles River), 321 ± 12 g ı = 31), were fasted overnight prior ;ery but were given free access to Vessels not supplying the hindlimb gated (only one limb was perfused), and the aorta and vena cava were then cannulated. More extensive details of the surgical preparation can be found elsewhere (10).

Perfusion apparatus. The environmental chamber and the arterial reservoir-oxygenator have previously been described by Zivin and Snarr (5). The perfusate was pumped at the desired flow rate by a variable speed microinfusion pump (Holter Model RL 175) from the arterial reservoir through a glass filter packed with Dacron. The Dacron was replaced at the beginning of every experiment and all tubing was flushed with 0.9% NaCl to remove loose particles of Dacron. A few centimeters beyond the filter, a bubble trap was inserted in the line. The perfusate was then pumped through the arterial cannula into the rat's hindlimb. The bubble trap contained a side arm connected to a Statham pressure transducer. Perfusion pressure was recorded on a Beckman RB Dynograph.

The venous effluent flowed from the vena cava into the venous cannula. This cannula passed outside the chamber and the venous perfusate flowed into a venous reservoir. Perfusate samples were taken from the arterial reservoir and venous cannual for analysis. The perfusate was not recirculated. An oxygen electrode (Beckman) was inserted in the venous line in preliminary experiments to determine if the limb could maintain a steady state rate of oxygen consumption.

Perfusion medium. The perfusion medium consisted of Krebs Ringer bicarbonate buffer, bovine albumin (Armour, fraction V), dextran (Sigma, MW 70,000–80,000), and washed dog erythrocytes (5). The artificial blood had a hematocrit of 32–34%, 2% albumin, 2.4% dextran, and 50–400 mg% glucose. Porcine insulin, 700 μU/ml, was also added to the perfusate. This dose of insulin should given muscle a near maximal glucose permeability (6).

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:served.

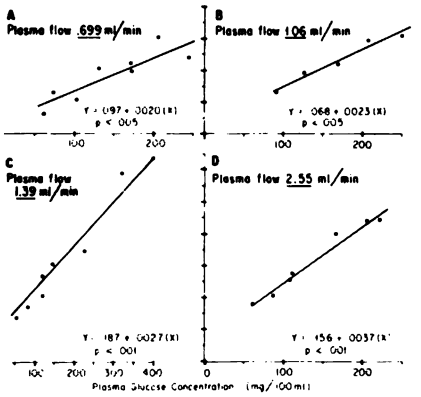
Analysis of perfusate. The glucose concentration in arterial and venous "plasma" samples was determined using a Technicon autoanalyzer employing the ferricyanide reduction method (11). The glucose uptake rate was calculated by multiplying the arteriovenous plasma glucose difference by the plasma flow rate. The plasma values were used in this calculation since it was found that 98% of the glucose added to the perfusate was recovered in the plasma several hours after adding the glucose, irrespective of whether the perfusate was at 37 or 4°. This recovery was found to be constant and independent of the exogenous glucose concentration. The reason the glucose did not penetrate the erythrocytes is unknown; however, it may be related to the fact that the cells were stored in an ACD solution and thus were exposed to a high glucose concentration for several days prior to use. There was no drop in the perfusate glucose concentration during its passage through the perfusion system, indicating no significant glucose utilization by the erythrocytes. The perfusate samples were centrifuged and analyzed for glucose immediately after the samples were taken.

All glucose uptake rates were expressed per 22.5 g of tissue, which was the average weight of the perfused limb tissue. The glucose uptake rates showed no correlation with leg weights over the narrow range of rat body weights employed.

Results. Development of a stable preparation. Preliminary studies were carried out in order to ascertain the stability of the preparation with respect to glucose uptake rate, venous oxygen tension, and vascular resistance. It was found that inclusion of insulin in nonrecirculated perfusate resulted in a preparation that reached a steady state rate of glucose uptake after 30 min and remained stable for an additional 270 min.

The venous oxygen tension was found to remain in the *in vivo* range, over a 5-hr perfusion period, and did not show a progressive increase as may be expected in a deteriorating preparation.

Vascular resistance (perfusion pressure/flow) remained stable for the first 3 hr of perfusion, but began to rise slowly after this time. Thus, the length of all experiments



2. Linear relationship between the rate of glu- ake and the plasma glucose concentration at w rates. Each point is an average of three determinations from one rat. Data shown at w rate are from two rats. Each point is an of three glucose determinations taken at 5-min during an experiment. The glucose concentra- changed 3–4 times in each experiment. The rations were randomized as to the time from of the experiment a glucose concentration was o be run. The flow rate in each experiment was stant.

nificantly different from zero ($P <$ at the four flow rates studied, indi- that at very low glucose concentra- the uptake rate is probably curvilin- owever, since no data were taken in nge, only the straight part of the were analyzed. In panel C, Fig. 2, it noted that the plasma glucose con- ion was raised substantially higher or the other three flow rates tested; the scale on the abscissa is slightly nt. It can be seen (Fig. 2C) that the uptake rate remains linearly related ma glucose concentration up to 400

ussion. It has been shown that both cose concentration and the flow rate ce the rate of glucose uptake by mus-

steady state glucose uptake rates re- in this study are a result of glucose ort across the capillary membrane and ort into the muscle. Data from this lo not permit separation of the com- of glucose transport due to muscle ane from that due to the capillary ane. However, the influence of the capillary on glucose transport is likely to be small compared to the influence of the muscle membrane on glucose uptake (12).

The glucose uptake vs plasma glucose concentration relationship was linear in the narrow range of glucose concentration chosen in this study (*in vivo* range). Daniel *et al.* (13) have shown that raising the concentration of glucose in the plasma will cause saturation of the mechanism by which glucose is transported into muscle. This group found the K_t (glucose concentration at which the glucose entry rates were half maximal) was about 600 mg/100 ml, whereas in the insulin-treated animal, the rate of glucose entry into the muscle rose in direct proportion to the plasma glucose concentration up to a concentration of at least 1440 mg/100 ml (13). Thus, in our study it is probable that the linear relationship between glucose uptake rate and concentration will hold well above the glucose concentrations tested.

The saturating relationship between glucose uptake and flow rate may be due to several possibilities. The first possibility is that as flow rate increases, the venous glucose concentration and interstitial glucose concentration approach a maximum, this being equal to the arterial concentration.

Another possibility to explain the saturating relationship between flow and uptake rate is arteriovenous shunting (14). It has been suggested that muscle may contain two vascular circuits situated in parallel, composed of vessels of two different types. It is hypothesized that one type, which receives most of the flow at low flow rates, is composed of capillaries where exchange takes place (14–16). As the rate of flow increases it is thought that more blood may be shunted through a parallel vascular circuit, this circuit being composed of vessels not thin enough for solute exchange. Thus, it may be that as flow rate increases, more blood transverses the nonexchangeable vessels, and glucose uptake rate reaches a maximum.

It is not possible to determine which of these hypotheses or combination of hypotheses is responsible for the relationship seen between flow rate and glucose uptake rate in the present investigation. But one

point is certain, the limitation of the flow-dependent increase in the rate of glucose uptake is not caused by saturation of a transport step at the muscle cell, for a saturable step would also be affected by an increase in arterial glucose concentration.

The hyperbolic relationship between blood flow and glucose uptake rate appears to be of physiological importance. The best estimate of blood flow in the femoral artery in the resting rat hindlimb is 1.4 ml/min for a 500-g rat (17) (leg wt about 30 g), which is a plasma flow of approximately 0.8 mg/min/30 g (assuming a hematocrit of 42%) or about 0.6 ml/min/22.5 g. At this flow rate, glucose uptake rate falls at the "knee" of the hyperbola (Fig. 1). Doubling the *in vivo* flow would by itself give a 25% increase in the rate of glucose uptake according to the results of this investigation. Thus, deviation from the resting flow rate *in vivo* should give corresponding changes in the rate of glucose uptake and may be partially responsible for the increased rate of glucose uptake seen during exercise. However, the rate of glucose uptake by skeletal muscle has been reported to increase as much as 10 times during exercise (18), and therefore an increase in blood flow rate would account for only a small part of the increased glucose uptake rate seen during exercise.

It should be pointed out that while in the rat it appears that increasing the blood flow beyond the *in vivo* resting blood flow will increase the glucose uptake rate, this may not be true for the skeletal muscle of all species. Indeed, it depends where on the curve (Fig. 1) the resting "operating point" falls for the skeletal muscle of each species.

In light of these results, the practice of maintaining a desired arterial pressure and allowing flow rate to change passively when doing metabolic studies on perfused rat muscle preparations should be reevaluated.

Summary. This investigation has defined

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 37-40 (1977)

ts of Ethanol on Bicarbonate-Stimulated ATPase, ATP, and Cyclic AMP in Canine Gastric Mucosa[1] (39598)

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ugh the stomach is one of the pri-
tes of action of ingested alcohol,
ve been few studies designed to ex-
e mechanisms by which alcohol af-
: gastric mucosa. Inhibition of the
ansport of chloride in the rat stom-
topical application of 20% ethanol
n demonstrated (1). Studies con-
on the isolated (2) and intact dog
(3) have also demonstrated inhibi-
active ion transport and acid secre-
model for active chloride transport
ı described by Kasbekar and Durbin
:h is operative through a bicarbon-
ulated ATPase, coupling chloride
:nt with bicarbonate in a manner
o ATPase-activated sodium and po-
transport. However, unlike the so-
us potassium ATPase, chloride is
:essary for maximal bicarbonate
activity (4). The only physiological
ich is known to stimulate this ATP-
ıicarbonate. Simon and Sachs (5)
gested that this enzyme may play a
bicarbonate transport in the pan-

:termine whether the ethanol-in-
hibition of chloride transport in the
ıucosa is mediated by inhibition of
·bonate-stimulated ATPase, the ef-
:thanol were evaluated on bicarbon-
ılated ATPase activity, *in vitro* and
ATP content, and *in vivo* cAMP
of dog gastric mucosa.

ds. Magnesium and magnesium
ırbonate-stimulated ATPase activi-
: determined according to Blum *et*
ısing crude homogenates of canine gastric mucosa. Gastric segments were resected from dogs fasted for 24 hr and anesthetized with 0.68 g/kg of chloralose-urethane (1:10). The mucosa was separated from the muscular portion of the stomach using glass slides. The mucosal portion was homogenized (1:10) in 0.25 *M* ice-cold sucrose with a ground glass tissue grinder, diluted, and analyzed within 1 hr. Reaction mixtures contained the following final concentrations: 100 m*M* Tris-HEPES (pH 8.2), 3 m*M* $MgCl_2$, 3 m*M* ATP, homogenate equal to 2.0 mg of tissue per ml of reaction mixture, and 20 m*M* $NaHCO_3$ when bicarbonate stimulation was evaluated. The samples were incubated at 37° and the reaction was terminated by pipetting 0.2 ml of the reaction mixture into 0.4 ml of 5% TCA. Denatured protein was removed by centrifugation and the quantity of inorganic phosphate released during the reaction was determined by the method of Buell *et al.* (7).

Effects of ethanol on ATP were evaluated using thin mucosal slices incubated in dog plasma gassed with 95% O_2 and 5% CO_2 at 37° with and without 20% ethanol (v/v). At varying time intervals, the tissues were removed, blotted, frozen in liquid nitrogen, pulverized, and homogenized in 5% TCA. Wet weights remained stable when three mucosal slices were incubated with 20% ethanol in dog plasma for 10, 20, and 90 min and sequentially weighed. ATP was quantitated according to the bioluminescent method of Stanley and Williams (8).

In vivo effects of ethanol on ATP and cAMP contents of dop gastric mucosa (fasted 24 hr) were evaluated using a chambered segment previously described by Mao *et al.* (9). A segment of the fundic gastric mucosa (approximately 36 cm^2) was mounted in a Lucite chamber with blood supply maintained intact. The chamber was divided into experimental and control sides.

investigations were supported by NIAAA
ıber 2 RO1 AA 00194-04.
hanbour is the recipient of Research Scien-
ıpment Award Number 5 KO2-AA-70463-

to whom reprint requests should be ad-

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rved.

After a 30-min exposure to alcohol, freeze-clamped biopsies were taken of the mucosa from each side. These tissues were pulverized in liquid nitrogen, homogenized in 1.0 ml of 5% TCA, and 0.10-ml aliquots were removed for determination of ATP. The homogenates were then purified on cation-exchange columns prepared according to Krishna *et al.* (10), and the cAMP was quantitated by the protein-binding assay described by Gilman (11).

Results. The effects of various concentrations of ethanol on Mg^{2+} and Mg^{2+} HCO_3^- ATPase activities are illustrated in Fig. 1. Both activities were inhibited 50% at 13.7 and 13.2% ethanol (v/v), respectively. Only a slight but insignificant degree of inhibition was observed with concentrations of 10% or less. The activities were inhibited 96% with concentrations of 20% ethanol. Figure 2 illustrates the effects of ethanol on ATP content in the tissue slice incubation studies. Ethanol significantly ($P < 0.05$) reduced ATP levels for all time intervals tested from 10 to 30 min. At 30 min the ATP content, after treatment with ethanol, was 93% less than the initial level (0 min). The ATP content of the control tissue at 30 min showed a 40% reduction compared to its initial value. Results were essentially the same whether expressed as wet tissue weight or dry tissue weight.

In vivo studies confirmed the *in vitro* observations. After a 30-min *in vivo* exposure to 20% ethanol, ATP content was reduced 44% as compared to the saline control side of the chambered mucosal flap (Fig. 3).

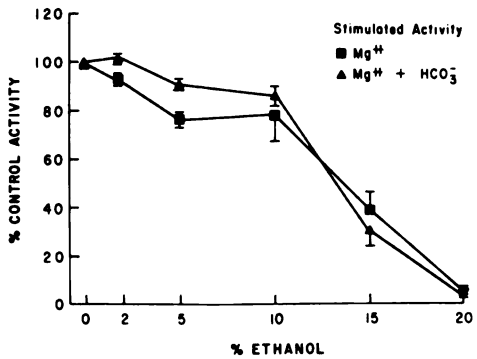


FIG. 1. Effects of various concentrations of ethanol on Mg^{2+}- and $Mg^{2+}HCO_3^-$-stimulated ATPase activities. Means ± SE for three experiments.

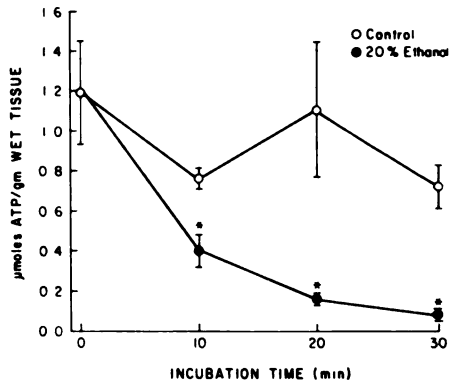


FIG. 2. Time-dependent effects of 20% ethanol on mucosal ATP content in tissue slice incubation studies. Asterisks indicate significant difference of $P < 0.05$ when compared with control at same time interval. Means ± SE for three experiments.

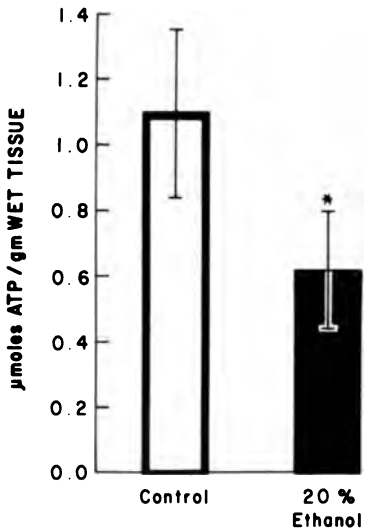
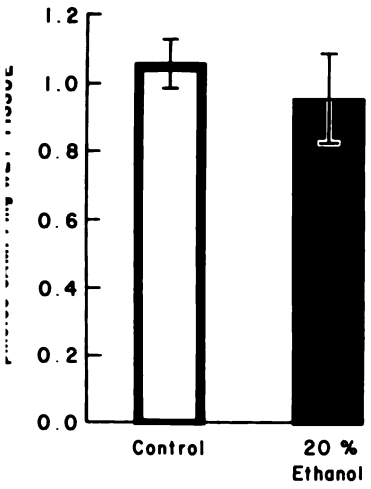


FIG. 3. *In vivo* effects of ethanol on gastric mucosal ATP content. Asterisk indicates significant difference of $P < 0.05$ when compared with control. Means ± SE for six experiments.

However, a 30-min exposure of the chambered flap to 20% ethanol did not significantly decrease cAMP content (Fig. 4).

Discussion. The demonstration of inhibition of magnesium and magnesium plus bicarbonate ATPases in the dog gastric mucosa by ethanol is in agreement with experiments conducted by Israel *et al.* (12), who showed a depression by ethanol of sodium plus potassium ATPase in rat and guinea pig brain. In addition, Tague and Shanbour



. *In vivo* effects of ethanol on cAMP content mucosa. Means ± SE for six experiments.

ve shown that ethanol inhibits adenyclase, the enzyme which converts) cyclic AMP, and phosphodiesterich converts cyclic AMP to 5'-AMP, change in cyclic AMP content which ably due to the greater activity of odiesterase as compared to adenylase.

finding that the gastric mucosal con-ATP is decreased both *in vitro* and *in* 20% ethanol is in agreement with studies by Carter and Isselbacher hese investigators demonstrated a e in small intestinal and liver ATP 18 hr after administering 7.5 g/kg of by stomach tube in the rat. They that the lowered ATP content was to ethanol metabolism by utilizing a g agent, 1 m*M* pyrazole. Similar obns have also been reported for the of ethanol on rat liver ATP content ker and Gordon (15) and Hyams and her (16). However, acute studies ted by French showed an increase in r ATP at 8 hr after ethanol adminiswhich returned to control levels at but a decrease in ATP content ocafter chronic ethanol feeding (17). crease in ATP content observed *in* well as *in vitro* could be an integral the mechanism by which ethanol active transport of H^+, Na^+, and Cl^- mammalian gastric mucosa (1-3). Electrophysiological studies have demonstrated that inhibition of active transport precedes permeability alterations which may lead to subsequent mucosal ulceration (1-3).

Inhibition by ethanol of active transport of sodium in frog skin has been demonstrated by Israel and Kalant (18). Mozsik (19) reported inhibition of Na^+K^+-dependent ATPase from human gastric mucosa by cAMP and 5'-AMP. Therefore, if ethanol stimulated adenylate cyclase activity, as reported by Greene *et al.* (20) for rat jejunum, the resultant increase in cAMP or metabolites could produce depressed active transport if ATPase enzymes are involved. However, since previous studies by Tague and Shanbour (13) and Puurunen and Karppanen (21) show inhibition of gastric adenylate cyclase by ethanol, rather than stimulation, and the present studies show no significant change in cAMP content, it is unlikely that ethanol-inhibited active transport of ions (1-3) in the gastric mucosa is mediated by cAMP.

Summary. Previous reports have demonstrated that ethanol inhibits acid secretion and active transport in the gastric mucosa. In the present studies, enzymes which have been implicated in gastric active transport (Mg^{2+} and Mg^{2+} HCO_3^- ATPases), as well as ATP and cAMP, were evaluated in response to ethanol. The activities of Mg^{2+} and $Mg^{2+}HCO_3^-$-stimulated ATPases were not altered at concentrations below 10% ethanol, but were significantly inhibited with 15 and 20%. Tissue slice incubations with 20% ethanol show significantly decreased ATP concentrations at 10, 20, and 30 min. Exposure of the dog gastric mucosa *in vivo* to 20% ethanol produced a significant decrease in ATP content but did not alter the cAMP level. These studies suggest that the ethanol-produced decrease in gastric ATP content may be involved in the inhibition of active ion transport observed with ethanol, but that cyclic AMP is probably not involved.

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Received May 24, 1976. P.S.E.B.M. 1977, Vol. 154

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 41-44 (1977)

Effects of Bethanechol on Intestinal Ion Transport in the Rat (39599)

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ıe nerves that supply the intestine ıucosal transport as well as motility? udies of Wright *et al.* (1), cutting the nic nerves to the small intestine of caused secretion that was enhanced cholinesterase inhibitor, physostig- nd that was reduced by atropine. ın has been elicited in the rabbit ı *vitro* with pilocarpine (2), and in jejunum with bethanechol (3). Re- [studied the effect of pilocarpine on ısport of water and electrolytes by small intestine (4). Pilocarpine is a y occurring alkaloid that is unlike ıoline in structure but which mimics arinic activity. In the present study, nined the effect of bethanechol, a nd that is similar in structure to ace- ıe, but which is inactivated at a ower rate. There are some interest- unexpected differences in the activi- he two compounds.

ɔds. Male Carworth rats weighing) g were fasted overnight and anes- by injecting pentobarbital (35 mg/ the peritoneal cavity. A 20-cm seg- proximal jejunum was cannulated end, washed with 20 ml of saline, ıhed with air. A 20-cm segment of ːum was similarly treated. A trache- tube was inserted. Solution was per- ıce through the segments with a sy- fusion pump at the rate of 0.41 ml/ ːing two 30-min periods, but fluid ected for study only during the last of each period. The input syringe, ː, and collection syringe attached to al cannula formed a closed system ıinimized leakage of CO_2. Before rfusion period, the intestinal lumen hed with the perfusion solution and hed with a mixture of O_2 and 5-6% s). At the end of each period, col- ıyringes were removed and capped, fluid remaining in the segments was flushed with gas and discarded. After the second period, the segments were removed, stripped of mesentery, and weighed.

The perfusion fluid had the following composition (m*M*): Na = 145, K = 5, HCO_3 = 25, Cl = 125, mannitol = 25. All solutions were gassed and had an initial pH of 7.42-7.43 and PCO_2 of 40-46 Torr. [1,2-^{14}C]Polyethylene glycol ([^{14}C]PEG) was used as a nonabsorbable marker to permit calculation of net water movement.

The pH and PCO_2 of the luminal fluid were determined soon after collection with a capillary pH electrode and PCO_2 electrode designed for small samples. Total CO_2 was measured with Natelson microgasometer. Sodium and potassium concentrations were measured with a flame photometer and chloride was determined with a coulometric chloridometer. The concentration of [^{14}C]-PEG (counts per minute per milliliter) was measured with a scintillation counter.

"Initial" chemical determinations were measured in samples of fluid obtained from the input syringes and samples of fluid for "final" determinations were obtained from the collection syringes after each collection period. Fluid remaining in the segments was discarded.

Net fluxes of ions and water were calculated as follows:

$$J_{net}^{ion} = V\left(ion_f \frac{PEG_i}{PEG_f} - ion_i\right) gWW^{-1}$$

$$J_{net}^{H_2O} = V\left(\frac{PEG_i}{PEG_f} - 1\right) gWW^{-1}$$

Ion_i and ion_f are the concentrations of the ion (micromoles per milliliter) measured in the initial and final samples; PEG_i and PEG_f are the specific activities of [^{14}C]PEG (counts per minute per milliliter) measured in the initial and final samples. V is the volume (milliliters) of perfusion pumped

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ɛrved.

into the segment in 20 min; gWW is the wet weight of the intestinal segment in grams. Thus, J_{net}^{ion} is expressed as micromoles × gWW^{-1} × $(20\ min)^{-1}$, and $J_{net}^{H_2O}$ is expressed as milliliters × gWW^{-1} × $(20\ min)^{-1}$. The signs preceding the net flux measurements indicate movement into (+) or out of (−) the lumen.

To measure transmural electrical potential difference (PD), bridges of saturated KCl in agar were used. One bridge contacted the fluid perfusing the intestinal lumen and the other was placed in the peritoneal cavity. Electrical contact with a voltmeter was made through calomel half cells. The voltage (mV) was recorded every 30 sec.

To determine the effect on PD, bethanechol was infused for 10-min periods at increasing rates of 0.24, 0.76, and 2.4 μmoles × kg^{-1} × min^{-1}. After each 10-min period of bethanechol infusion, isotonic saline was infused for 10 min to permit the PD to return to baseline values.

To assess the effect of parenteral bethanechol on ion and water movement, isotonic saline was infused intravenously during the first 30-min perfusion period, and bethanechol was infused during the second at the rate of 2.4 μmoles × kg^{-1} × min^{-1}.

To determine whether bethanechol affected ion and water movement when it was in contact with the external surface of the lumenal cell membrane, bethanechol was added to the intestinal perfusion fluid in the second period in a concentration of 0.5 m*M*. Intravenous bethanechol infusion at 2.4 μmoles × kg^{-1} × min^{-1} at the end of 30 min should produce an extracellular fluid concentration of about 0.5 m*M* assuming that none entered the cells, that there was no degradation, and that extracellular fluid volume was 15% of body weight. Hence, in all likelihood, the concentration of bethanechol in the intestinal perfusion fluid was higher than that attained in the extracellular fluid by intravenous infusion.

The statistical significance of differences between means was determined with a Student's *t* test for paired samples.

Results. Dose response of PD. The negative lumenal PD of the jejunum increased as the infusion rate of bethanechol was increased (Table I). The lowest PD of the control period immediately preceding the infusion was used as the baseline value to calculate the magnitude of the change. At the infusion rate used in the studies of electrolyte and water transport, 2.4 μg × kg^{-1} × min^{-1}, the mean lumenal PD became more negative by 3.3 mV. The PD attained its maximum 6–8 min after the start of the infusion and remained the same throughout the remainder of the period. Because, in the transport studies, the study periods began 10 min after the commencement of the infusion of bethanechol, it is likely that the changes induced by bethanechol were in a steady-state during the period of the study.

At the highest dose, all rats salivated copiously, tears appeared, and most of them urinated or defecated. At the intermediate dose, there was some salivation but at the lowest dose, none of these changes were observed.

Effect of intravenous bethanechol. In the jejunum, bethanechol reduced absorption of Na from 44.0 to 13.8 μmoles × gWW^{-1} × 20 min^{-1} and bicarbonate absorption diminished from 47.7 to 20.9 μmoles × gWW^{-1} × 20 min^{-1} (Table II). Bethanechol increased the final perfusate pH from 7.06 to 7.20 and reduced the final PCO_2 from 70 to 60 Torr.

In the ileum, bethanechol increased the final perfusate pH from 7.40 to 7.43. The large standard deviations in the net movements of Na and Cl in the control studies were caused by one rat which secreted Na and Cl at high rates. If the data from that rat are excluded from the statistical analysis (and $N = 7$), the SD's for Na and Cl net

TABLE I. EFFECT OF BETHANECHOL ON JEJUNAL TRANSMURAL ELECTRICAL PD.[a]

	Bethanechol (μmoles/kg × min^{-1})		
	0.24	0.76	2.4
Δ PD (mV)	−0.3±0.27	−2.1±1.08 ***	−3.3±1.82 **

[a] The mean (± SD) change in jejunal electrical potential difference (PD) caused by intravenous infusion of bethanechol at three different rates. The sign indicates lumenal electrical negativity. The change in magnitude of the PD is related to the PD of the control period immediately preceding the bethanechol infusion; $n = 5$. ** $P < 0.005$; *** $P < 0.001$.

TABLE II. EFFECTS OF BETHANECHOL ON INTESTINAL TRANSPORT.[a]

	Jejunum		Ileum	
	Control	Bethanechol	Control	Bethanechol
Na				
$\bar{X}$	−44.0	−13.8*	−0.1	−6.2
SD	34.3	34.5	61.6	14.8
K				
$\bar{X}$	−0.93	−0.28	+0.96	+0.69
SD	1.37	2.37	1.93	0.73
Cl				
$\bar{X}$	+7.7	+21.0	−24.7	−30.1
SD	20.2	38.9	48.7	17.7
HCO_3				
$\bar{X}$	−47.7	−20.9**	+19.6	+30.7
SD	16.4	21.7	32.2	12.2
H_2O				
$\bar{X}$	−0.27	−0.12	+0.09	−0.01
SD	0.29	0.28	0.33	0.01
Final pH				
$\bar{X}$	7.06	7.20***	7.40	7.43**
SD	0.07	0.08	0.02	0.02
Final PCO_2				
$\bar{X}$	70	60***	49.5	47.7
SD	7	8	2.2	2.8

[a] For measurements of net movement of electrolytes and water, signs indicate movement into (+) or out of (−) lumen. Rates of net transport of electrolytes or water are expressed as micromoles or milliliters × gram wet ght of intestine^{-1} × 20 min^{-1}. The values of pH and PCO_2 (Torr) are those measured in the collected perfusion d; $N = 8$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ınsport during the control period are re- ced to 17.8 and 16.5, respectively, but ɛ means still do not differ significantly.

Effect of intralumenal bethanechol. Be- ınechol did not affect the net movement electrolytes and water, the pH, or the CO_2 of the perfusate.

Discussion. The increase in the electrical gativity of the lumenal PD is similar to ıt observed in the dog jejunum with be- ınechol (3), in the rat jejunum *in vivo* th acetylcholine (5), or pilocarpine (4), d *in vitro* with acetylcholine or neostig- ne (5). Part of this change may be attrib- ɛd to an enhanced net movement of Cl o the lumen (3, 4), or to a reduction in CO_3 absorption and H secretion (4), but ıer unknown factors may contribute.

The changes in transport induced by be- ınechol are generally similar to those ısed by pilocarpine which differs from thanechol in structure. However, whereas pilocarpine reduced the secretion of H and caused secretion of Na, K, Cl, and HCO_3, the effect of bethanechol was more selectively on Na, HCO_3, and H. This is additional evidence of a close link between the absorption of Na and the secretion of H in the rat jejunum (6).

It is difficult to estimate the contribution of reduced transit time to the changes in net ion movement in this preparation. Three findings support the view that the role of transit time was not predominant, however: (i) the changes in PD that reflect a direct effect of bethanechol on mucosal ion transport, (ii) the absence of major changes in ion transport in the ileum where contractility would also be expected to increase, and (iii) the absence of a reduction in Cl secretion, for Cl secretion should diminish with transit time.

The changes in net transport evoked by pilocarpine are quantitatively similar in the

jejunum and ileum (4), whereas the major effect of bethanechol is on the jejunum, implying that the muscarinic receptor of the ileum is less accessible or is less responsive to bethanechol. Since intralumenal bethanechol had no effect on transport, the receptor in the sensitive jejunum does not lie on the external surface of the plasma membrane. Although bethanechol might affect extraintestinal sites that could influence mucosal function through hormones or extrinsic nerves, the responsible receptors are probably located in the basolateral cell membrane or in the intrinsic nerves.

The present study shows that the systems that transport salt and water in the jejunum of the rat have the capacity to respond to the acetylcholine analogue, bethanechol. It remains to be demonstrated that this has any physiological significance.

Summary. In studies of rat jejunum, parenteral bethanechol increases the transmural PD, and decreases Na and HCO_3 absorption, the rate of H secretion, and the final

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ɔnal Blood Flow in Dogs with Normal and Spontaneously Elevated Blood Pressure[1] (39600)

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ge number of physiological and ological stimuli alter the distribu- ɔlood flow within the kidney. Re- ıbe *et al.* (1) investigated the influ- ɔlood pressure per se on the distri- f blood flow within the kidney. In ıdies, renal perfusion pressure was y means of aortic clamping. No ef- intrarenal distribution were de- 'hen renal perfusion pressure was d from control values (142 ± 5 mm 05 mm Hg. In contrast, reduction ure below 100 mm Hg increased tical flow and decreased outer cor- /.

: present time, no information is concerning the distribution of w within the kidney as a function of taneously occurring blood pressure. e, we studied the distribution of ıl blood flow utilizing radiolabeled ıeres in 60 nondiuretic, pentobarbi- hetized mongrel dogs as a function spontaneous mean arterial blood . These studies are of interest in our previous report that bilateral cclusion with the resultant increase perfusion pressure results in selec- eases in inner cortical vascular re- (2).

ds. Sixty mongrel dogs of either sex ɛg) were anesthetized with pento- (30 mg/kg iv). The animals were ɔmized, and positive pressure venti- ıs applied with a Harvard Appara- animal respirator. A solution con- *para*-aminohippurate (PAH) and e in saline was administered iv at ıin/kg body wt via a PE-100 cath- rted into the right external jugular ɔod pressure was recorded with a aph pressure transducer (P-1000A) and recorder (Four-A) via a PE-100 catheter inserted into the right femoral artery. The left femoral artery was also cannulated with a PE-100 catheter, and the catheter tip was advanced into the thoracic aorta several inches above the renal arteries. This left femoral artery catheter served for collection of arterial blood samples and injection of microspheres (see below for details). Renal venous samples were obtained through Kifa catheters (U.S. Catheter Corp.) positioned in the right and left renal veins via the femoral veins. Right and left ureters were cannulated with PE-100 tubing.

When urine flow had stablized (1.5 to 2.5 hr), two consecutive clearance periods were performed with midpoint arterial and renal venous samples. Approximately 200,000 ^{169}Yb-labeled microspheres (15 ± 5 μm; 3M Corp.) were then rapidly injected into the thoracic aorta. A number of different experimental manipulations followed these control estimates of blood flow distribution, but the results are not germain to the current report. At the termination of each experiment both kidneys were removed, weighed, and placed in 10% formaldehyde solution overnight. Saggital kidney sections (approx 0.5 cm) were then manually cut. Two cortical zones, approximately equal in thickness, were easily discernible: a dark outer cortex and a light inner cortex. With the exception of the superior and inferior renal poles, the entire outer cortex was then removed, weighed, and radioactivity was determined. Any outer cortex remaining on the renal pieces was removed, and the entire inner cortex was dissected free, weighed, and counted. Total microsphere content in each kidney was calculated from the sum of outer and inner cortical counts plus the counts of residual material. Radioactivity was determined on a Packard gamma scintillation counter (Model 3002).

Creatinine concentration was estimated

tudy was supported by NIH Grant No.

977 by the Society for Experimental Biology and Medicine
rved.

by the picric acid method of Folin and Wu (6), and PAH by the diazotization procedure of Bratton and Marshall (4). Na^+ and K^+ concentrations were measured with flame photometry (Advanced Instruments Corp.).

Renal blood flow (RBF) was calculated from PAH clearance, renal extraction of PAH, and hematocrit values. Blood flow per gram outer (f_{oc}) or inner (f_{ic}) cortex was calculated according to the method of Katz *et al.* (8).

Data shown in each figure and table represent means and standard errors. However, the reported linear regression equations were derived from individual data points rather than the means.

Results. The mean blood pressures of the 60 anesthetized dogs reported in this study ranged from 90 to 180 mm Hg. The number of dogs at each mean arterial blood pressure and corresponding RBF, total renal resistance (R_T) and glomerular filtration rate (GFR) values are summarized in Table I. Renal resistance is expressed as peripheral resistance units per gram kidney weight. The dogs were characterized by a poor but nonetheless significant inverse relationship between RBF per gram kidney weight and mean arterial blood pressure. The linear regression equation for RBF and blood pressure was: $RBF = 0.015\ \overline{BP} + 6.64$ ($r = -0.239$, $P < 0.01$). Furthermore, evaluation of the relationship between total renal resistance and mean arterial blood pressure also revealed a significant correlation. The least squares line was $R_T = 0.30\ \overline{BP} - 9.32$ ($r = 0.464$, $P < 0.001$). Glomerular filtra-

TABLE I. MEAN ARTERIAL BLOOD PRESSURES, RENA
RENAL VASCULAR RESISTANC

Number of dogs	Arterial mean blood pressure	RI
3	90	5.4 :
4	110	5.0 :
9	120	4.8 :
10	130	4.7 :
11	140	4.5 :
11	150	5.3 :
10	160	4.4 :
2	180	4.1 :
$\bar{X}$	137 ± 20	

[a] Values are means ± SE.

The wide range of intrarenal blood flow tribution noted above should have optimd evaluation of the potential involvement this physiological parameter in the con-

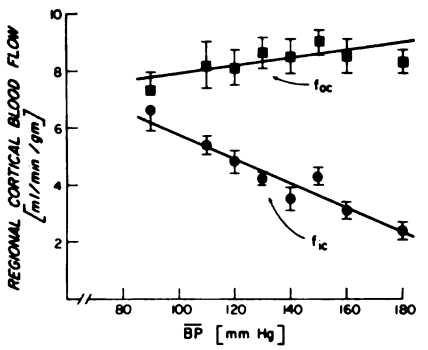

FIG. 1. Inner (f_{ic}) and outer (f_{oc}) cortical blood w per gram inner or outer cortex (± SE) is shown as unction of mean arterial blood pressure. Linear ression lines are indicated ($f_{ic} = -0.04\,\overline{BP} + 9.99, r$ $-0.534, P < 0.001$; and $f_{oc} = 0.12\,\overline{BP} + 6.55, r$ $2, P > 0.1$).

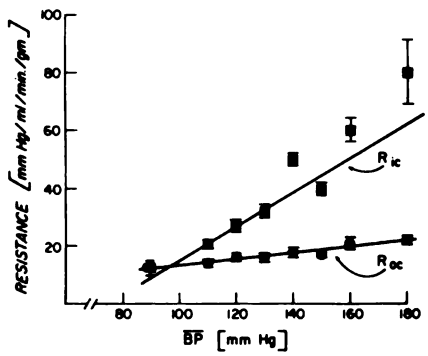

FIG. 2. Calculated values for inner (R_{ic}) and outer $_{ic}$) cortical resistance (± SE) are shown as function blood pressure. Linear regression equations (R_{ic} = '0 $\overline{BP}$ − 54.96, $r = 0.64$; $P < 0.001$; and $R_{oc} = 0.10$ + 3.14, $r = 0.34$, $P < 0.001$) are drawn.

trol of electrolyte excretion. Amounts of Na^+ ($U_{Na}V$) and K^+ (U_KV) excreted per gram kidney weight as well as fractional excretion of Na^+ (FE_{Na^+}) and K^+ (FE_{K^+}) are shown in Table II. Neither fractional nor absolute Na^+ excretion per gram kidney weight showed significant correlations with inner cortical flow, outer cortical flow, or with the ratio of inner to outer cortical blood flow (IC/OC). Indeed, $U_{Na}V$ per gram kidney weight was essentially constant, averaging 1.95 μEq/min/g kidney wt ± 0.78 (SE). In contrast, U_KV (microequivalents per minute per gram kidney weight) showed a poor but significant inverse correlation with the ratio of inner to outer cortical blood flow ratio ($U_KV = 0.27$ IC/OC + 0.66, $r = -0.24$, $P < 0.01$). However, this correlation between absolute potassium excretion and flow distribution may be fortuitous since FE_{K^+} and the IC/OC ratio were not significantly correlated.

Discussion. The renal hemodynamic changes associated with hypertensive disorders are generally characterized by a decrease in renal blood flow and an increase in renal vascular resistance (3, 5, 7). The results obtained in this study show a similar inverse correlation between mean arterial blood pressure and total renal blood flow (Table I). Furthermore, calculated values for renal vascular resistance were approximately 2.5 times greater in those dogs at 180 mm Hg vs those at 90 mm Hg blood pressure (Table I).

As may be seen in Fig. 1, the distribution of intrarenal blood flow changed considerably as a function of mean arterial blood pressure. These changes in intrarenal blood flow were largely ascribable to differences in

TABLE II. Na^+ AND K^+ EXCRETION AT DIFFERENT BLOOD PRESSURES.[a]

Mean arterial blood pressure (mm Hg)	$U_{Na}V$ (μEq/min/g kidney wt)	U_KV	FE_{Na} %	FE_K %
90	0.6 ± 0.4	0.33 ± 0.05	0.5 ± 0.30	17 ± 4
110	1.9 ± 0.7	0.46 ± 0.07	1.9 ± 0.60	20 ± 2
120	1.4 ± 0.3	0.52 ± 0.04	1.8 ± 0.30	29 ± 2
130	1.9 ± 0.4	0.51 ± 0.04	2.4 ± 0.60	29 ± 2
140	2.1 ± 0.5	0.62 ± 0.07	2.7 ± 0.90	29 ± 2
150	2.8 ± 0.6	0.53 ± 0.04	3.1 ± 0.80	26 ± 3
160	1.8 ± 0.5	0.48 ± 0.04	2.3 ± 0.70	23 ± 2
180	1.4 ± 0.2	0.64 ± 0.06	1.2 ± 0.01	24 ± 3

[a] Values are means ± SE.

inner cortical hemodynamics. Thus, dogs with mean arterial pressures of 180 mm Hg were characterized by inner cortical blood flows which were approximately 1/3 that found in dogs with mean arterial pressures of 90 mm Hg. In addition, regional vascular resistance (Fig. 2) was significantly higher in the inner cortex compared to the outer cortex at elevated blood pressures.

The changes in intrarenal hemodynamics which were observed in the current study could be related to differences in myogenic, hormonal, or neurogenic factors in the inner cortex compared to the outer cortex. However, the results of Abe *et al.* (1) appear to rule out a myogenic factor since they have shown that changes in renal perfusion pressure alone between approximately 140 and 105 mm Hg did not influence intrarenal blood flow distribution. Renal perfusion pressure in their studies was controlled by means of aortic clamping. As was noted in the Results, we found a significant inverse correlation between inner cortical flow and mean arterial blood pressure in the 140- to 110-mm Hg range.

With respect to the other possibilities, i.e., hormonal or neurogenic factors, further experiments will be required to elucidate which variable accounts for the intrarenal hemodynamic events described in this study. Nonetheless, it is of interest to note that the current results are similar to the changes in intrarenal blood flow distribution which occur with bilateral carotid occlusion (2), a maneuver known to increase renal sympathetic nerve tone (9).

Finally, while it seems reasonable to assume that some parameter of renal function would be related to intrarenal flow distribution and that the substantial flow distribution differences we observed in these studies would have optimized elucidation of this function, no differences, at least with respect to Na^+ and K^+ excretion, were found (Table II). The possibility exists that other parameters of renal function are dependent on intrarenal flow distribution or that the functional changes are relatively small and cannot be detected during acute studies.

Summary. Intrarenal blood flow distribu-

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ıd Bile Acid Synthesis after Interruption of the Enterohepatic Circulation: Evidence against Hormonal Mediation[1] (39601)

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ɔtion of the enterohepatic circula-
reation of a bile fistula or ileal
causes a fall in serum bile acid
2) and a five- to tenfold increase in
ɜynthesis in the experimental ani-
or man (6–10). To explain this
ɔn, several authors have suggested
ıcid synthesis is regulated by the
f bile acids returning to the liver

culated that another explanation
ɔossible. The concentration of bile
ɔrtal or systemic blood might influ-
elease of a hormone which in turn
ɜulate hepatic bile acid synthesis.
ıis, we have carried out experi-
ıg the *in situ*-isolated perfused ca-
and compared the effect of blood
nal dogs with that of dogs with
bile acid synthesis on the rate of
ynthesis by the perfused liver. As
we also perfused livers from dogs
ased bile acid synthesis with blood
ıal dogs.

ls and methods. The experimental
featured the isolated perfused
scribed by Urquhart (12) in which
f a small dog is perfused *in situ* by
ood from a large dog. Details of
ısion technique have been pub-
ɔviously (13). The experimental
ıs a 2-by-2 Latin Square: livers
l dogs with or without ileal resec-
tion were perfused by large dogs with or without ileal resection.

The animals used were mongrel dogs who had been treated for intestinal worms, were vaccinated against rabies and canine distemper, and had been on regular kennel diet for at least 3 weeks. Twelve days prior to study, all animals underwent laparotomy under pentobarbital anesthesia, and a modified Thomas cannula was positioned in the third part of the duodenum to serve as a sham operation in the animals not resected. Continuity of the intestine was reestablished with an end-to-end anastomosis in the larger dogs and a side-to-side anastomosis in the small dogs. The extent of resection was confirmed by weighing the excised gut at the time of surgery and the remainder of the gut at the conclusion of the study. All animals used made an uneventful recovery from surgery and were within 15% of their preoperative weight at the time of the study.

In each experiment a dog of 20–30 kg (pump dog) was used to provide the circulatory system to support the isolated liver of a second dog (donor liver dog) of 4–8 kg. The isolated liver was left *in situ* and perfused via the portal vein with blood from the femoral artery of the pump dog. The inferior vena cava of the donor liver dog was cannulated and liver venous outflow was returned to the femoral vein of the pump dog. The hepatic artery of the donor liver dog was ligated. Blood flow to the perfused liver was maintained at a constant value in the range of 0.8–1.5 ml/g liver or 30 ml/kg body weight donor liver dog, by adjusting portal pressure in the range of 5–20 cm H_2O. The cystic duct was ligated and a cannula placed in the common bile duct for bile collection.

Bile was collected for half-hour periods for at least 7 hr in each study. Portal inflow pressure and portal blood flow were moni-

ed in part by Public Health Service Re-
ts Numbers AM 6908 and AM 16770.
address: Department of Medicine, Austin
niversity fo Melbourne, Melbourne, Aus-
offman was a FA Hadley Travelling Fellow
ıiversity of Western Australia and an Aus-
ɜrican Educational Foundation Scholar.
reprint requests to Dr. Alan F. Hofmann,
ɔlogy Unit, Mayo Foundation, Rochester,
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ɜd.

tored continuously and recorded every 15 min, as was systemic blood pressure in the pump dog. An intravenous infusion of dextran was used as required to maintain systemic blood pressure. Oxygen tension, Hb, and O_2 saturation was measured in the inflow and outflow blood of the perfused liver on three occasions, 2 hr apart. This allowed calculation of hepatic oxygen utilization which was always in the range of 1.5–4 mmol/kg liver/min. Tissue was taken for histologic examination at the end of each experiment and all tissue was within normal limits.

Bile was analyzed for total bile acid concentration in each sample by a modification of the 3α-steroid dehydrogenase technique of Talalay (14).

The bile acid pool of the pump dog was labeled with [^{14}C]cholyltaurine 24 hr prior to the experiment on two occasions. By estimation of the specific activity of gallbadder bile acid from the pump dog and the bile acid secretion by the donor liver dog, we were able to show that the pump dog contributed less than 5% of the secreted bile. Therefore, we considered the bile acid secreted after the first hour to be newly synthesized bile acid.

The four possible combinations of resected and sham-operated dogs were tested: 10 experiments were performed using two sham-operated dogs; 2 experiments with a sham-operated donor liver dog and an ileal-resected pump dog; 2 experiments with an ileal-resected donor liver dog, and a sham-operated pump dog; and 2 experiments in which both pump and donor liver dogs underwent ileal resection.

The perfused liver was weighed at the end of the experiment and the rate of bile acid secretion is expressed as micromoles per kilogram of liver per minute to allow comparison of livers of differing weights.

Results. In the control experiments, the rate of bile acid secretion was quite steady by the end of the first hour of perfusion, but a slow decline occurred during the next 7 hr (Fig. 1). Essentially the same pattern of bile acid secretion was seen when the liver from a sham-operated dog was perfused by the blood of an animal with an ileal resection (Fig. 2). However, if the donor liver dog had had an ileal resection, the rate of bile acid secretion was considerably elevated above base line whether the pump dog had an ileal resection (Fig. 3) or was sham-operated (Fig. 4). In all studies, the rate of bile acid secretion beyond the first hour was considered to be an estimate of the rate of bile acid synthesis, since in an animal with a gallbladder such as the dog, most of the bile acid pool is excluded when the liver is prepared for perfusion.

Discussion. These experiments lend no support to the concept of a hormonal substance controlling bile acid synthesis. Previous work with this model has demonstrated a response to glucagon within 2 hr (12), and an isolated perfused rat liver has been shown to respond to cortisol growth

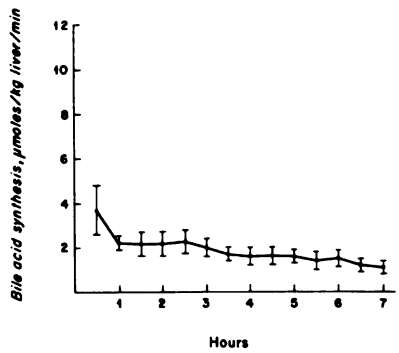

FIG. 1. Time course of bile acid synthesis (M ± SE) in the *in situ*-perfused canine liver in 10 dogs. Both the donor and pump dog had undergone a sham ileal resection.

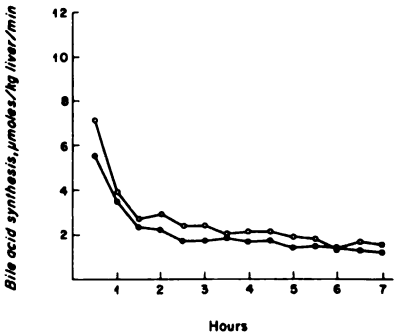

FIG. 2. Time course of bile acid synthesis in the *in situ*-perfused canine liver of two dogs each of whom had undergone a sham ileal resection; the pump dog had undergone ilial resection. Each curve represents a single study.

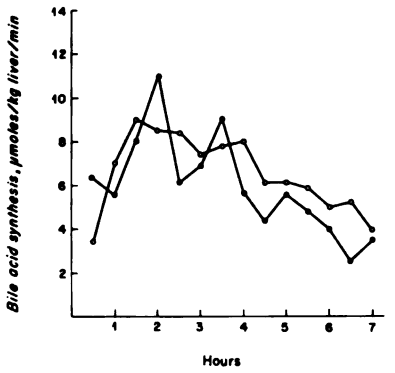

FIG. 3. Time course of bile acid synthesis in the *in* *tu*-perfused canine liver of two dogs each of whom ad undergone ileal resection; the pump dog had also ndergone ileal resection. *Each curve represents a sin-e study.*

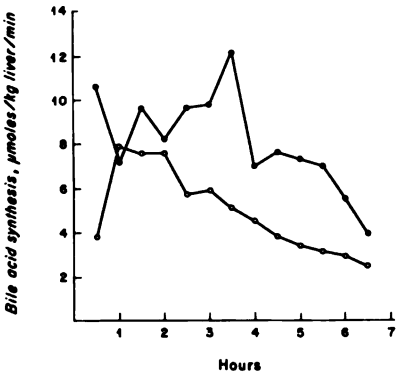

FIG. 4. Time course of bile acid synthesis in the *in* *tu*-perfused canine liver of two dogs, each of whom ad undergone ileal resection; the pump dogs had a am ileal resection. *Each curve represents a single udy.*

ormone and triiodothyronine within 2 hr 15). Thus, a response by the perfused liver n these experiments might have been ex-ected within 7 hr although the possibility of substance of longer latent period has not een excluded. We think it unlikely that the ailure to observe a response by the per-used liver is attributable to loss of capacity or bile acid secretion, since we have shown reviously that this preparation will secrete physiological bile acid load (0.22 mmole/ r) quantitatively even after 7 hr of perfu-ion (13).

Other workers (16) have implied that it is nore likely that it is the concentration of bile acids within the hepatocyte that determines the rate of bile acid synthesis. Thus, bile acid synthesis falls during fasting (17) despite a decreased return of bile acids to the liver, perhaps because of increased concentrations of bile acids in the liver cell in association with decreased bile acid secretion.

Summary. Experiments were carried out using an *in situ*-perfused canine liver to test whether a hormone is responsible for the striking increase in bile acid synthesis occurring after interruption of the enterohepatic circulation by ileal resection. Large dogs with ileal resections or sham operations were used as pump dogs to perfuse the livers from small dogs with sham surgery or ileal resection. Bile acid synthesis during hours of observation was not influenced by the type of pump dog. Since most known hormones act rapidly on the isolated perfused liver, the experiments suggest that a hormonal substance is not responsible for the striking increase in bile acid synthesis observed when the enterohepatic circulation is interruped by removal of the ileum.

We acknowledge the skillful technical assistance of Susan B. Coffin and Lawrence L. Aarhus. Dr. R. G. Shorter reviewed the liver histology.

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Received June 22, 1976. P.S.E.B.M. 1977, Vol. 154

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tomy between the Release of Bone Marrow Granulocytes and Stem Cells (39602)

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ministration of endotoxin to a f experimental animals including ts in a release of marrow granulo-) possibly mediated by a humoral med leukocytosis-inducing factor anges in peripheral blood granulo- ophage *in vitro* colony-forming :) after endotoxin injection paral- nges in blood mature granulocyte th marked elevations seen 6 hr toxin (6, 7). That these peripheral ations of CFC are also due to a m the bone marrow is suggested rease in marrow CFC with either or no change in splenic CFC seen -h interval after endotoxin injec- . When endotoxin is administered to CF1 or C57 black mice, a " or resistance develops to the ny-stimulating factor (CSF), elec- ct of endotoxin although granulo- nue to be released from the bone). In the present study, we have the effect of endotoxin preinjec- ie peripheral blood granulocyte esponse to endotoxin.

. The *in vitro* soft agar culture is a modification of a double-layer iique (10) which has previously ted (8). The present studies were t over a 2-year time interval dur- he final concentration of fetal calf increased gradually from 13 to ing this time, there was a progres- se in plating efficiency of periph- ; however, qualitatively the re- periments were similar. Whole heparinized peripheral blood (Panheparin without preservative, Abbot Laboratories; final concentration approximately 25 units/ml) was added directly to the agar-culture medium mixture and plated; colonies of over 50 cells were counted on Day 10. This time interval of growth was selected because at this time there was enough lysis of red cells to allow for accurate colony counts. Peripheral blood granulocyte values were determined on individual blood samples anticoagulated initially with versenate (K_2EDTA), but in later experiments granulocyte counts were determined on pooled heparinized blood: the results were the same with either method. Bone marrow cells were flushed from four to five tibias with single-strength Eagle's medium and pooled. The nucleated cell concentrations of the pooled suspensions were determined and 25,000 marrow cells were plated in the soft agar culture technique. The methods employed were the same as those for peripheral blood CFC except that the colonies were counted on Day 8. In the present experiments, control CF1 mice (Carworth Farms) were injected for 7 days with 10 μg of *Salmonella typhosa* endotoxin (Difco 3946-10) intraperitoneally (ip): 24 hr after the seventh injection, mice from control and tolerant groups were injected with either 0.1 ml of saline or 5 μg of *S. typhosa* endotoxin ip, and 6 hr later the peripheral blood CFC and granulocyte values were determined. In some experiments, peripheral blood CFC were also determined at earlier time points after the injection of either saline or endotoxin. Each experimental group at each point consisted of from four to nine individual mice.

Marrow and peripheral blood differentials were measured as previously reported (8). At least 100 cells per animal were

ly supported in part by grants from the irt and Lung Institute (HL 5600 and HL om the General Research Branch (NIH nd PHS (Grant No. HL-07142-02). Leukemia Society of America.
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counted for peripheral blood. Paint-brush bone marrow smears were made from individual femurs and from 410 to 515 cells were counted for each femoral smear.

Statistical significance was determined utilizing the Wilcoxon rank order test (two tails).

Results. Figure 1 shows the peripheral blood granulocyte values in control and tolerant mice 6 hr after the ip injection of 0.1 ml of saline or 5 μg of *S. typhosa* endotoxin ip. Both endotoxin-injected groups show significant increases in blood granulocytes. Table I presents the values of differentiated marrow cells expressed as the absolute number per tibia. Absolute increases in total granulopoiesis inthe tolerant groups are apparent and a significant decrease in marrow mature neutrophils in both of the endotoxin-injected groups is clearly shown, indicating that the increase in peripheral blood granulocyte values in these animals was indeed due to a release of granulocytes from the bone marrow storage pool.

Figure 2 shows the peripheral blood CFC

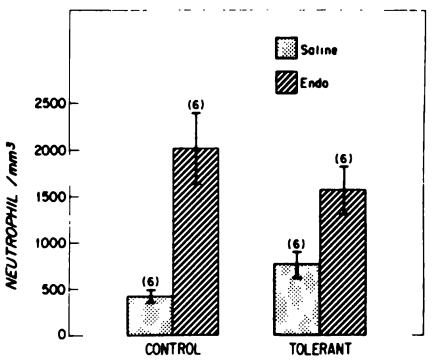

FIG. 1. The peripheral blood neutrophils (band and polymorphonuclear) per cubic millimeter ± 1 SE from control and tolerant mice 6 hr after either 5 μg of endotoxin or 0.1 ml of saline ip. Numbers in parenthesis represent the number of separate experiments from which these data were derived.

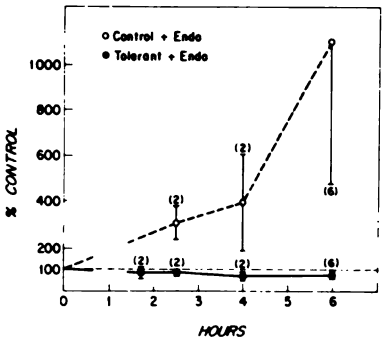

FIG. 2. Peripheral blood CFC from tolerant and control mice at varying time intervals after 5 μg of endotoxin expressed as a percentage of the saline-injected controls ± 1 SE. The numbers in parenthesis represent the number of separate experiments from which these data were derived.

values in tolerant and control mice at varying times out to 6 hr after 5 μg of endotoxin ip. The values at 6 hr are from the same experiments presented in Fig. 1. There are marked increases in the numbers of peripheral blood CFC in control mice 6 hr after the injection of 5 μg of endotoxin as compared to the saline-injected control mice ($P < 0.05$). The peripheral blood CFC levels in tolerant mice 6 hr after 5 μg of endotoxin were not significantly different from those seen in the saline-injected tolerant mice. There were also no increases in peripheral blood CFC in tolerant mice 1¾, 2½, and 4 hr after endotoxin, indicating that an earlier release of marrow CFC had not occurred in the tolerant mice. The absolute numbers of CFC per milliliter of peripheral blood ± 1 standard error of the mean (SE) in control and tolerant mice 6 hr after ip injection with saline were 1686 ± 733 and 1460 ± 473, respectively. The absolute numbers of CFC per milliliter of blood ± 1 SE 6 hr after 5 μg of endotoxin in control and tolerant mice were 5692 ± 1970 and 1175 ± 381, respectively.

TABLE I. TIBIAL CELL VALUES ($\times 10^{-6}$).[a]

Group	Proliferative granulocytes	Metamyelocytes	Bands	PMN	Lymph	RBC
Control Saline	0.707 ± 0.230	0.826 ± 0.028	1.910 ± 0.513	2.18 ± 0.356	3.574 ± 1.02	3.491 ± 0.846
Control Endo	0.539 ± 0.189	0.884 ± 0.014	1.263 ± 0.454	0.485 ± 0.235	3.404 ± 0.275	3.641 ± 0.802
Tolerant Saline	1.117 ± 0.142	1.664 ± 0.298	2.867 ± 0.543	5.910 ± 1.24	0.761 ± 0.266	0.983 ± 0.095
Tolerant Endo	1.231 ± 0.249	1.609 ± 0.075	2.885 ± 0.217	3.13 ± 0.189	1.006 ± 0.547	1.580 ± 0.114

[a] The number of various cell types per tibia in control and tolerant mice 6 hr after either 5 μg of endotoxin or 0.1 ml of saline. These data are derived from two experiments included in the data in Fig. 2, and each point is based on data derived from 13–18 individual femoral smears.

experiments the number of CFC were determined. The mean num- C per tibia ± 1 SE in tolerant mice 5 μg of endotoxin was 142 ± 17% ne-injected tolerant mice. The ab- ues for tibial CFC ± 1 SE in toler-) hr after either endotoxin or saline 165 ± 18,120 and 40,200 ± respectively. These differences statistically significant.

ion. In the present studies tolerant e continued to release marrow tes in response to endotoxin, but r showed increases of peripheral C after endotoxin. The increases eral blood CFC seen after endo- ction of CF1 mice were presum- ndary to marrow release, since in studies utilizing the same strain of id dose of endotoxin, we found a iarrow CFC without any decrease CFC over the 6 hr after endotoxin ccordingly, the absence of an in- peripheral blood CFC in endo- cted tolerant mice was presumably ailure of release of CFC from the The absence of decrease in tibial e endotoxin-injected tolerant mice nsistent with this conclusion. It cknowledged that changes in mar- numbers after endotoxin may be to a number of mechanisms other ation from the marrow and thus interpreted with caution. Changes CFC may be secondary to altera- uxes between the marrow pluripo- cell (CFU) compartment, the CFC ient, and the differentiated granu- npartment. The decrease in mar- seen after endotoxin injection of ice could, in part, be due to differ- in the marrow granulocytic path- the suggestion of an increase in C (not statistically significant) in nt endotoxin-injected mice could an increased differentiation of CFC or perhaps a decreased out- the CFC compartment into the ited marrow compartment or pe- lood pool. In addition, as we have c label for the CFC, we cannot sess peripheral blood turnover or n and thus changes in the latter cannot at present be ruled out as contributing to our results. It seems likely, from the studies sited above that we were, in fact, dealing with a marrow release of stem cells, a phenomenon well documented in irradiated leg-shielded animals (11, 12).

These data imply that there may be basic differences in the manner in which granulocytes and stem cells are released from the bone marrow. There is evidence that a humoral entity termed leukocytosis-inducing factor may mediate endotoxin-induced granulocyte release (4, 5); perhaps stem cell release is mediated by a separate factor or a direct effect of endotoxin. Alternatively, differences intrinsic to the cell could underlie differences in marrow release. Lichtman and Weed (13, 14) have presented evidence that polymorphonuclear neutrophils (PMN) may be more deformable and adhesive than their less mature, morphologically recognizable progenitors, and it is possible that similar differences exist between PMN and CFC. In this case, the CFC would be less deformable and adhesive than PMN and thus less likely to exit from the bone marrow. If there were a simple quantitative decrease in the release stimulus, as might occur with the development of endotoxin tolerance, a decrease in the release of marrow CFC might be expected prior to any demonstrable decrease in granulocyte release.

The above studies do not deal directly with the mechanism of the stem cell release seen after endotoxin injection. In normal CF1 mice injected with 5 μg of endotoxin, there is a temporal correlation between increases in serum CSF levels and stem cell release (7, 9). Tolerant CF1 mice injected with 5 μg of endotoxin fail to release stem cells and do not show increases of serum CSF levels over the 6 hr after injection. This suggests that CSF may play a role in stem cell release.

Summary. Control CF1 mice injected ip with 5 μg of *S. typhosa* endotoxin release marrow granulocytes and granulocyte-macrophage *in vitro* colony-forming cells (CFC). Mice repetitively injected with 10 μg of endotoxin daily for 7 days and then challenged on Day 8 with 5 μg of endotoxin release granulocytes but not CFC from the marrow, indicating that the mechanisms

governing release are dissimilar for the two cell types.

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Received March 26, 1976. P.S.E.B.M. 1977, V 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 57-59 (1977)

Pulmonary Heart Disease Induced by Disenecioyl Dehydroretronecine: A Semisynthetic Pyrrolizidine Alkaloid Pyrrole[1] (39603)

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tion of the pyrrolizidine alkaloids phytotoxins found in plants of the *Senecio*, *Crotalaria*, and *Heliotro*auses severe hepatic and cardiopul lesions in man and animals (1). Pyrne alkaloids themselves appear to be ly nontoxic; however, as a result of lism to PA pyrroles the toxicity is ly enhanced (2). Pyrrolic metabouse pulmonary fibrosis, pulmonary nsion, and cardiac hypertrophy (3,

past it has been difficult to evaluate chanisms of action of the PAs and rrole metabolites primarily because ifficulty in preparing them in a radioform. Recently this difficulty has rtially circumvented by the synthesis radioactive semisynthetic pyrrolizikaloid, [^{3}H]disenecioyl retronecine the present report, data are prewhich show that disenecioyl dehynecine, the pyrrole of disenecioyl ine, produces lung lesions similar to naturally occurring PA pyrroles.

ials and methods. Disenecioyl dehynecine (DSDR) was prepared as ly described (6). Briefly, retrone-β-hydroxy-1-hydroxymethyl-1,2-de-α-pyrrolizidine) was acylated with l chloride (3,3-dimethyl-acryloyl) to yield the semisynthetic PA diseretronecine (di-3,3-dimethyl acrylic er of retronecine). This was then with $KMnO_4$ to disenecioyl dehynecine (di-3,3-dimethyl acrylic acid dehydroretronecine).

y-five male ARS-Sprague-Dawley rats, initially weighing 45-55 g, were randomly divided into a control group of 10 rats and an experimental group. Control rats received 0.05 ml of dimethylformamide (DMF) iv into the left jugular vein while the experimentals were injected via the same route with 15 mg of DSDR/kg body weight (21 mg DSDR/ml DMF). Rats were sacrificed 14, 21, 28, and 35 days postinjection by cannulating the trachea and infusing the lungs with 4% formalin in saline at 3 cm of water pressure (8). The lungs and heart were removed *en block* and placed in jars containing fresh Carstair's fixative (8). Other tissues were fixed in 10% buffered formalin. After fixation, the hearts were cut and the right ventricle (RV), left ventricle (LV), and interventricular septum (IVS) were individually weighed. The right ventricular ratio (RVR) was calculated by the formula: RVR = RV g/(LV + IVS g). A RVR greater than 0.50 was considered as indicative of right ventricular hypertrophy (RVH) (9). Sections from the lung and other major organs were stained with hematoxylin and eosin. Additional lung sections were stained with Carstair's stain for fibrin (8).

Results. Three experimental rats which died spontaneously on Days 6 and 9 postinjection and three experimental animals sacrificed 14 days postinjection did not develop RVH and showed only a diffuse, mild septal hyperplasia and fibrosis in the lungs. The nine experimental animals sacrificed 21 to 35 days after injection had an $\bar{X}$ RVR of 0.58, of these, eight had RVH. The eight control rats sacrificed at the same time period had an $\bar{X}$ RVR of 0.26. Table I summarizes the pulmonary alterations found in the animals sacrificed 21 to 35 days postinjection. In contrast to controls, extensive arteriolar intimal hypertrophy and hyperplasia

vestigation was supported in part by U.S. lth Service Grants No. HL-10941, No. CA-. GM-00130, and No. RR-00167 from NIH. nter Publication No. 16-027.

1977 by the Society for Experimental Biology and Medicine rved.

were commonly seen (Fig. 1). These lesions, while focal, were seen distributed throughout the lungs of animals with RVH. Other pulmonary alterations included increased numbers of alveolar macrophages, septal edema, interstitial fibrosis, and accumulation of fibrin in the walls of the arterioles. Typically, the right myocardium of animals with RVH showed myocyte hypertrophy, interstitial edema and hypercellularity, and focal necrosis (Fig. 2). Structural alterations were not seen in the left ventricle or interventricular septal myocardium.

Discussion. This and a previous report from our laboratory (6) demonstrate that DSDR causes hepatic and cardiopulmonary lesions similar to those produced by the naturally occurring PA pyrroles dehydromonocrotaline (4) and dehydroretronecine (7). Like the latter two, DSDR would appear to exert its vasotoxic effects on the endothelium of the first microvascular system it enters. Here it initiates a sequence of changes leading to pulmonary microvascular occlusion, resulting in increased resistance to pulmonary blood flow and the subsequent development of right heart hypertrophy. Associated with these changes are septal thickening due to edema and hypercellularity, increased numbers of alveolar macrophages, and, in the right heart, myocyte hypertrophy and focal myocytolysis. Since only minimal isolated alterations were seen in the veins of the lung, it may be assumed the semisynthetic PA pyrrole failed to escape the lung. We feel that the cardiac lesions seen in this study are therefore secondary to the pulmonary lesions.

TABLE 1. LESIONS DEVELOPING IN RATS AFTER A SINGLE IV INJECTION OF DISENECIOYL DEHYDRORETRONECINE.[a]

Lesion	Treatment: DSDR[b] 15 mg/kg	Treatment: Control 0.05 ml DMF[c]
Arteriolar intimal hypertrophy and hyperplasia	8[d] (9)[e]	0 (8)
Interstitial cellular hyperplasia	8 (9)	0 (8)
Interstitial fibrosis	7 (9)	0 (8)
Septal edema	6 (9)	0 (8)
Fibrin in wall of arterioles	6 (9)	0 (8)
Periarteritis	3 (9)	0 (8)

[a] Animals sacrificed 21 to 35 days following injection.
[b] DSDR = Disenecioyl dehydroretronecine.
[c] DMF = Dimethylformamide.
[d] Number of animals with lesion.
[e] Number of animals injected.

The lung lesion produced by the PAs differs slightly from those caused by the PA pyrroles. As with the pyrroles, there is thickening of the alveolar septae, increased alveolar macrophages, and occasionally a periarteritis (3). Unlike the pyrroles, the PAs cause medial hypertrophy of the small muscular arteries of the lung. This hypertrophy has been attributed to the development of pulmonary hypertension (10). However,

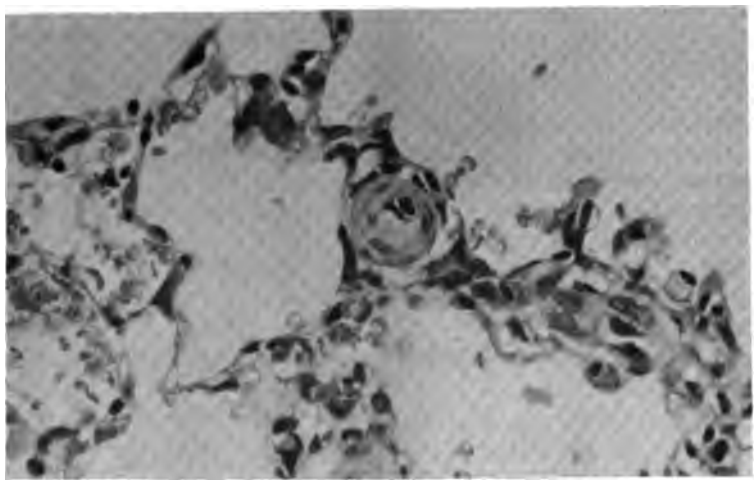
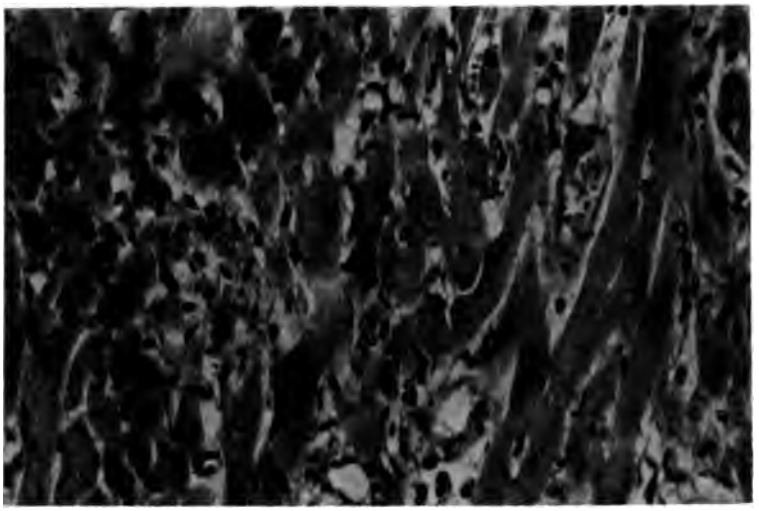


FIG. 1. Effect of disenecioyl dehydroretronecine on the lung in a rat sacrificed 28 days postinjection. The rat heart ratio of 0.63. Note the occluded arteriole and the septal hypercellularity. H & E stain. × 270.



2. Right ventricle from a rat sacrificed 21 days after being injected with disenecioyl dehydroretronecine. nal had a right ventricular ratio of 0.66. This photograph demonstrates the hypertrophy of myocytes, al hypercellularity, and parenchymal collapse seen in the rats which developed right ventricular hypertro- & E stain. × 290.

present reported study, the develop- of cor pulmonale in the experimental curred without medial hypertrophy. he medial hypertrophy of the pulmo- teries seen after PA intoxication may to a different mechanism than simply velopment of pulmonary hyperten- ossibly the parent alkaloid or some PA metabolite, like the dehydropyr- ne alcohols, e.g., dehydroretrone- ther alone or synergistically, initiates dial changes. Certainly the reaction PA metabolites with muscle cells is an hed fact since rhabdomyosarcomas roduced in a large percentage of rats lehydroretronecine (11).

nary. Rats injected with disenecioyl oretronecine, a recently synthesized thetic pyrrolizidine alkaloid pyrrole, ed cardiopulmonary lesions similar e caused by naturally occurring PA s. These alterations included pulmo- lema, alveolar edema and septal fi- occlusion of the pulmonary arteri- crease in alveolar macrophages, and art hypertrophy.

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Received June 25, 1976. P.S.E.B.M. 1977, Vol. 154.

Redistribution of Renal Cortical Blood Norepinephrine I

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It has been suggested that redistribution of renal blood flow within the cortex of the kidney alters sodium excretion (1, 2). The mechanisms by which this intrarenal distribution of renal blood flow may be regulated have not been elucidated. The purpose of the present study was to investigate the effect of two renal vasoconstrictor stimuli, renal nerve stimulation (RNS) and norepinephrine (NE), on the intrarenal distribution of renal blood flow, and to locate the cortical sites involved in regulation of intrarenal blood flow distribution.

The isolated blood-perfused canine kidney was used so that perfusion pressure (PP) and renal blood flow (RBF) could be controlled. In addition, the use of the isolated organ system eliminated systemic reflexes which otherwise might have altered the effect of RNS or NE.

Materials and methods. The method for isolated perfusion has been described fully in previous reports (3, 4). Kidneys were obtained from pentobarbital-anesthetized (30 mg/kg) male mongrel dogs weighing 18–23 kg. Within 2-min of removal, the kidneys were perfused with 800–900 ml of autologous heparinized blood at a constant temperature of 37°. The systolic pressure was maintained at 130 mm Hg as control pressure by adjusting RBF with a pulsatile pump (Waters Co.). For those experiments involving renal nerve stimulation (RNS), the renal nerves were removed with the kidneys. To accomplish this, the renal nerves were isolated as they approach the aorticorenal ganglion and cut distal to the ganglion. After perfusion of the kidney was begun, the nerves were secured in bundle fashion and

[1] This study was supported by grants from the U.S. Public Health Service, Grant No. NHL 12677 from the National Heart and Lung Institute, and by the Wisconsin Heart Association.

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after vasoconstriction, intrarenal ion of blood flow was again mea- iis always occurred within 15 min of rol measurement). The intensity of constriction was adjusted by alter- oltage of the stimulator or the dose ıfusion. Two studies were done on :ys in each study. In the first, the ;trictor stimulus was increased to erfusion pressure from 130 mm Hg to approximately 170 mm Hg sys- :n PP was adjusted back to 130 mm lic by reducing RBF with the pump. second study, the vasoconstrictor was again adjusted to increase PP ximately 170 mm Hg systolic. Per- ressure was left at this level; thus, ıained constant.

ical analysis of the data was per- ısing Student's *t* test for paired ob- ıs (8).

s. The functional characteristics of ted kidney during control periods ı in Table I. Renal blood flow was to that measured in anesthetized ıt glomerular filtration rate (clear- :reatinine) was about 30% less (9). ated kidney reabsorbed about 98% ltered sodium and 97% of the fil- ter, slightly less than the kidney *in* : isolated kidney concentrated the ıese results are similar to previously d data from this laboratory utilizing ted kidney model (3).

ffects of RNS and NE on total RBF vn in Table II. RNS and NE were in all cases to increase systolic pres- n 130 mm Hg to approximately 170 . In those experiments where the was subsequently returned to 130 with the perfusion pump, RNS and ificantly reduced RBF by 28 and espectively. In those experiments where the pressure was left at 170 mm Hg, RNS and NE had no significant effect on RBF. Thus, two experimental conditions (reduced RBF and constant RBF) were created during which the effects of RNS and NE on the intrarenal distribution RBF were measured.

Figure 1 shows the data for RNS and its effect on outer and inner cortical flow and on the intracortical distribution of blood flow for both experimental conditions (reduced RBF and constant RBF). RNS which significantly reduced RBF (first panel of Fig. 1, solid lines) also significantly reduced absolute flow to the outer cortex (from 108 ± 8 to 82 ± 9 ml/min) and to the inner cortex (from 39 ± 6 to 24 ± 5 ml/min). Although flow was reduced in both areas, the reduction was greatest in the inner cortex as exemplified in the significant increase in fractional flow (percentage of total RBF) to the outer cortex (from 75 ± 2 to 79 ± 2%) and significant decrease in fractional flow to the inner cortex (from 25 ± 2 to 21 ± 2%) (second panel of Fig. 1, solid lines). RNS which did not alter RBF (first panel

TABLE I. FUNCTIONAL CHARACTERISTICS OF TED KIDNEYS DURING CONTROL PERIODS (N = 15).

GFR (ml/min)	Fractional excretion of Na (%)	Fractional excretion of H_2O (%)	U/P osmolality
20.9 ± 1.0	2.6 ± 0.67	3.3 ± 0.59	1.57 ± 0.082

± SE.

TABLE II. SYSTOLIC PRESSURE AND RENAL BLOOD FLOW DURING RENAL NERVE STIMULATION (RNS) AND NOREPINEPHRINE INFUSION (NE).

	Systolic pressure (mm Hg)		Renal blood flow (ml/min)	
	C	E	C	E
RNS	130	130	147 ± 13[a]	106 ± 14*
NE	130	130	167 ± 13	128 ± 14*
RNS	130	170	155 ± 15	156 ± 18
NE	130	170	194 ± 16	189 ± 18

[a] Mean ± SE. N = 11 for each series of experiments.

* $P < 0.005$.

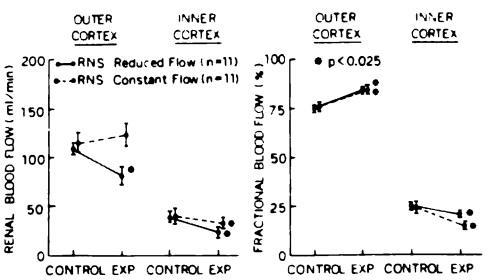

FIG. 1. Effect of renal nerve stimulation (RNS) on renal cortical blood flow distribution.

of Fig. 1, dashed lines) did not alter absolute flow to the outer cortex, although a tendency to increase was noted (from 115 ± 10 to 124 ± 14 ml/min). Inner cortical flow was significantly reduced (from 40 ± 7 to 32 ± 5 ml/min). This resulted in a significant increase in fractional flow to the outer cortex (from 76 ± 3 to 80 ± 2). Thus, when RNS decreased RBF, flow was reduced to both the outer and inner cortex with a redistribution of fractional blood flow from inner to outer cortex. When RBF was maintained constant in the face of RNS, only the inner cortex had a reduction in flow while the outer cortex had a tendency to increase flow, again with a redistribution of fractional blood flow from inner to outer cortex. These data demonstrate that RNS induced a greater vasoconstriction in the inner than outer cortical vasculature.

NE infusion produced the same pattern of results as RNS. This is shown in Fig. 2. NE infusion, which significantly reduced total RBF (first panel of Fig. 2, solid lines), reduced absolute flow to the outer cortex (from 116 ± 9 to 94 ± 11 ml/min) and to the inner cortex (from 51 ± 6 to 34 ± 5 ml/min). This resulted in an increase in fractional flow to the outer cortex (from 70 ± 2 to 74 ± 2%) and a decrease in fractional flow to the inner cortex (from 30 ± 2 to 26 ± 2%) (second panel of Fig. 2, solid lines). NE infusion which did not change RBF (first panel of Fig. 2, dashed lines) did not alter absolute flow to the outer cortex, although a tendency to increase was observed (from 133 ± 11 to 138 ± 13 ml/min). Inner cortical flow was decreased significantly from 61 ± 7 to 51 ± 7 ml/min. NE, with no change in RBF (second panel of Fig. 2, dashed lines), significantly increased fractional flow to the outer cortex (from 69 ± 2 to 73 ± 2%) and significantly decreased fractional flow to the inner cortex (from 31 ± 2 to 26 ± 2%).

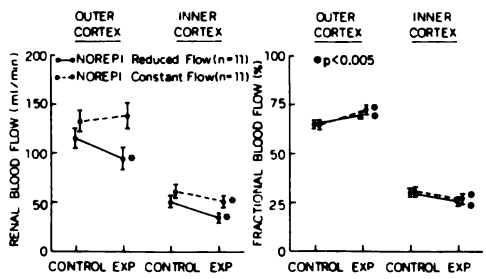

FIG. 2. Effect of norepinephrine infusion (NE) on renal cortical blood flow distribution.

NE thus produced similar effects as RNS, with a redistribution of fractional blood flow from the inner to the outer cortex in both conditions, when RBF was reduced and when RBF was not changed. It is apparent that NE, like RNS, has a greater vasoconstrictor effect on the inner cortical vasculature than on the cortical vasculature.

Discussion. In the present study, the use of the isolated perfused kidney permitted the evaluation of vasoconstriction on intrarenal hemodynamics under two experimental conditions. In the first series of experiments, vasoconstriction decreased RBF and perfusion pressure (PP) was held constant (130 mm Hg). These conditions reflect the *in situ* situation which may occur during the natural release of vasoactive hormones or increased sympathetic activity. Under these conditions, both RNS and NE caused a relative redistribution of RBF from the inner half of the cortex to the outer half of the cortex. Absolute flow to both areas was decreased, but there was a relatively greater decrease in the inner cortical flow. This suggests that the inner cortical vasculature responded to the stimuli with a greater vasoconstriction, although both RNS and NE caused vasoconstriction throughout the cortex.

In the second series of experiments, RNS and NE induced vasoconstriction elevated PP to approximately 170 mm Hg. Perfusion pressure was permitted to remain elevated as RBF was maintained constant. Under these conditions it is possible to define more clearly the sites of relative vasoconstriction. In a parallel circuit such as we have in the kidney, when RBF is constant any vasoconstriction will direct flow away from the area of greater resistance into an area of less resistance. Both RNS and NE directed flow from the inner half to the outer half of the cortex. In fact, under these conditions there was an increase in absolute flow to the outer cortex while decreasing flow to the inner cortex. This demonstrates that the vasoconstriction induced by RNS and NE is greatest

asculature supplying the nephrons of
r half of the cortex.
have been reports that alpha-adre-
timulation may not produce homo-
s vasoconstriction throughout all of
hrons. Pomeranz *et al.* (2) suggested
NS, induced either by bilateral ca-
gation or splanchnic nerve stimula-
creased outer cortical and increased
ortical and outer medullary blood
drenergic stimulation also has been
ed to cause a redistribution of blood
vay from the outer renal cortex in
with heart failure and in experimen-
els of sodium retention (1).
ntrast to these results, Stein *et al.*
d Rector *et al.* (11) have failed to
trate any effect of RNS (10) or NE
the intrarenal distribution of blood
he disparate results obtained by the
investigators are most probably ex-
by the different technique used to
the distribution of blood flow. In
s laboratory (1, 2) the gas-washout
was used, while in Stein's labora-
), 11), the radioactive microsphere
was used. As reviewed by Stein *et*
the gas-washout method measures
rtical flow instead of outer cortical
hile the microsphere method distin-
between the different areas within
ex. Thus, a decrease in outer cortical
measured by gas-washout actually
a decrease in total cortical flow.
r present study, both RNS and NE
total cortical flow in agreement with
washout studies (1, 2). In addition,
rospheres in the present study have
trated that within the cortex itself,
d NE result in a relative redistribu-
flow from the nephrons in the inner
the cortex to those of the outer half
cortex. Our study does differ from
f Stein and colleagues (10, 11) who
ed the microsphere method, but
no change in distribution of flow
RNS (10) or NE (11). The reason for
ering results is not clear. There are,
r, two possibilities. One is the possi-
tence of a different response occur-
an isolated organ system as com-
vith that occurring *in situ*. If this is
e reason for the difference needs to
be investigated. The second reason may be the different level of stimulus used. In the present study, RNS and NE (250–500 ng/min) reduced RBF by 28 and 23%, respectively. While in the *in situ* experiments, RNS reduced RBF 40% (10) and NE (4 μg/min) reduced RBF 49% (11). These may possibly explain the differing results; however, further study is needed to clarify the differences.

It is evident from the data presented here that both RNS and NE can cause a redistribution of renal blood flow within the cortex and that this redistribution is from inner to outer cortical nephrons.

For a redistribution of blood flow to occur, there must be a differential response of the renal vasculature to vasoactive stimuli. Through the use of radioactive microspheres various investigations have been able to demonstrate a redistribution of renal blood flow between inner and outer cortical nephrons in response to a variety of vasoactive stimuli (3, 13–15).

Renal vasodilation with bradykinin (13) and prostaglandins (3) in the isolated perfused kidney, and acetycholine (14) and dopamine (15) *in situ* have been reported to redistribute fractional blood flow from outer to inner cortex. This redistribution is the opposite of that demonstrated in the present paper with vasoconstriction. These results indicate that there was a greater vasodilation of the inner cortical vasculature than the outer cortical vasculature, resulting in a redistribution of flow from outer to inner cortical nephrons.

The data from the present paper (vasoconstriction) and previous investigations (vasodilation) suggest that the inner cortical vasculature is more responsive to vasoactive stimuli than is the outer cortical vasculature. Thus, the vessels supplying the inner cortical nephrons are primarily responsible for the regulation of flow between inner and outer cortical nephrons. This differential response to vasoactive stimuli of the vasculature in the superficial and deep cortex represents an important mechanism by which the intrarenal distribution of blood flow can be regulated.

Summary. The effects of renal nerve stimulation (RNS) and norepinephrine infusion

(NE) on the intrarenal distribution of renal blood flow were studied in the isolated blood-perfused kidney. Both RNS and NE caused a redistribution of fractional blood flow from the inner half to the outer half of the renal cortex. Both vasoconstrictor stimuli resulted in a relatively greater vasoconstriction of the inner cortical vasculature. It is concluded that the inner cortical vasculature is more responsive to vasoactive stimuli and is the important locus for regulation of the distribution of renal cortical blood flow.

The authors wish to thank the staff of Allen Bradley Medical Science Laboratory for their help in completing this project.

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Received June 22, 1976. P.S.E.B.M. 1977, Vol. 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 65-68 (1977)

cts of Dietary Carbohydrate on Sodium Cyclamate Toxicity in Rats Fed a Purified, Low-Fiber Diet (39605)

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sive literature is available indicating ımber of chemicals, drugs, and food s, when incorporated in purified, r diets, induces toxic effects in rats e at dosages which have little if any effect when fed with a natural food ation (1-5). Supplements of the ıutrients, either alone or in combi- when fed with the purified, low-fiber re without protective effect, but the fects were largely counteracted by ents of various dietary fiber-con- materials (3-6). Purified cellulose ıowever, when fed at a level compa- the fiber content of the above sup- s, had little if any protective effect. ıve findings suggest that some die- :rs present in a natural food stock /ere effective in counteracting the ects induced by certain dosages of chemicals, drugs, and food addi- ıasmuch as the purified diets indi- ıove contained dextrose or sucrose source of dietary carbohydrate, the natural food stock ration con- tarch and other complex polysac- as the major source of dietary car- tes, the question arose as to the type of carbohydrates present ıral food stock ration may not have ted to the protective effect of the tion in animals fed dosages of var- micals, drugs, and food additives ere toxic when fed in conjunction purified, low-fiber diets indicated n the present communication, data :nted on the comparative effects of low-fiber diets, containing sucrose, , dextrin, or cornstarch as the f dietary carbohydrate, on the re- ıf immature rats fed toxic amounts nnutritive sweetener, sodium cycla-

lure and results. Sixty male rats of gue-Dawley strain were selected at an average body weight of 52.7 g (range 49-56 g) and were divided into five comparable groups of 12 animals each. One group was fed a basal purified, low-fiber diet; the remaining groups were fed a similar diet + 5% sodium cyclamate[1] but differed as to the source of dietary carbohydrate. The composition of the experimental diets is indicated in Tables I and II. Animals were placed in metal cages with raised screen bottoms (three rats per cage) and were provided the test diets and water *ad libitum*. Animals were fed daily, and all food not consumed 24 hr after feeding was discarded. Body weight was recorded weekly. Feeding was continued for 14 days or until death, whichever occurred sooner.

In agreement with previous findings (3, 6), the addition of sodium cyclamate at a 5% level to the basal purified, low-fiber diet containing sucrose as the source of dietary carbohydrate (Diet II), resulted in a highly significant retardation in weight gain, lack of grooming, varying degrees of alopecia, and extensive diarrhea with watery and mushy stools. Of 12 rats fed the latter diet, 10 succumbed during the experimental period of 14 days. Similar findings were obtained in rats fed sodium cyclamate in conjunction with the dextrose-containing diet (Diet III), with 9 of 12 rats succumbing during the experimental period of 14 days. In contrast to the above, a marked improvement occurred in rats fed sodium cyclamate in conjunction with the dextrin- or cornstarch-containing rations. Ten of the 12 rats fed the dextrin-containing diet (Diet IV) and all 12 rats fed the cornstarch-containing diet (Diet V) survived the experimental period of 14 days. Gain in body weight (particularly dur-

[1] The sodium cyclamate employed in the present experiment was kindly provided by Dr. Ronald G. Wiegand of Abbott Laboratories, North Chicago, Illinois.

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rved.

ing the first week of feeding) was significantly retarded in rats in all groups fed sodium cyclamate compared to those fed the basal purified diet (Diet I). During the second week of feeding, a marked increment in body weight occurred in all surviving rats fed the cyclamate-containing diets. This was particularly marked in rats fed the cornstarch-containing diet whose gain in body weight during the second week of feeding did not differ significantly from that of rats fed the basal-purified, cyclamate-free diet.

TABLE I. COMPOSITION OF EXPERIMENTAL DIETS.[a]

Dietary components	Diet (%)				
	I	II	III	IV	V
Sodium cyclamate	0	5	5	5	5
Sucrose	66	61			
Dextrose			61		
Dextrin				61	
Cornstarch					61
Casein[b]	24	24	24	24	24
Salt mixture[c]	5	5	5	5	5
Cottonseed oil	5	5	5	5	5

[a] To each kilogram of the above diets were added the following vitamins: thiamine hydrochloride, 5 mg; riboflavin, 5 mg; pyridoxine hydrochloride, 5 mg; calcium pantothenate, 50 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; *para*-aminobenzoic acid, 200 mg; inositol, 400 mg; biotin, 1 mg; folic acid, 5 mg; vitamin B_{12}, 150 μg; 2-methyl,1,4-naphthoquinone, 5 mg; choline chloride, 2 g; vitamin A, 5000 U.S.P. units; vitamin D_3, 500 U.S.P. units; and alpha-tocopheryl acetate, 100 mg. The vitamins were added in place of an equal amount of dietary carbohydrate.

[b] Vitamin-Free Test Casein, Teklad Test Diets, Madison, Wisconsin.

[c] Mineral Mix, U.S.P. XVII, Teklad Test Diets, Madison, Wisconsin.

Extensive diarrhea and varying degrees of alopecia were manifest in rats fed the dextrin-containing diet. With the exception of a smaller size and slight diarrhea, rats fed the cornstarch-containing diet appeared grossly normal at the termination of the experiment. Food consumption was not determined for rats in the various groups for diarrhea in rats fed the cyclamate-containing diets contaminated the food and interfered with accurate determinations of food consumption data; the latter was particularly true for rats fed the sucrose- and dextrose-containing diets. It was readily apparent, however, from gross inspection of the food cups, that the amount of food consumed by rats fed the sucrose- and dextrose-containing rations (Diets II and III) was markedly less than that of rats fed the dextrin- and cornstarch-containing rations (Diets IV and V). Inasmuch as sodium cyclamate was present in all the above rations at a 5% level, animals with the largest food consumption also consumed the largest amount of sodium cyclamate. Findings indicate that the greater weight increment and lower mortality of rats fed the dextrin- and cornstarch-containing diets occurred despite the fact that these animals ingested more sodium cyclamate than did those on the sucrose- or dextrose-containing diets.

Discussion. Present findings indicate that the source of dietary carbohydrate significantly affected the response of immature male rats fed a purified, low-fiber diet containing 5% sodium cyclamate. When the source of dietary carbohydrate was sucrose

TABLE II. EFFECTS OF SOURCE OF DIETARY CARBOHYDRATE ON THE WEIGHT INCREMENT AND SURVIVAL OF IMMATURE MALE RATS FED A PURIFIED, LOW-FIBER DIET + 5% SODIUM CYCLAMATE.[a]

Dietary group	% Sodium cyclamate in diet	Dietary carbohydrate	Average gain (g) in body wt after following days of feeding:		% Survival after 14 days of feeding
			7 Days	14 Days	
I	0	Sucrose	35.8 ± 1.9[b]	78.6 ± 2.8	100
II	5	Sucrose	5.3 ± 2.1 (4)	18.5 ± 2.2 (2)	16.7
III	5	Dextrose	6.2 ± 4.8 (7)	32.3 ± 7.4 (3)	25
IV	5	Dextrin	4.2 ± 3.7	27.2 ± 8.0 (10)	83.3
V	5	Cornstarch	9.8 ± 3.5	48.7 ± 5.2	100

[a] The average initial body weight of rats in the various groups was 52.7 g. The values in parentheses indicate the number of animals which survived and on which data are based when this number is less than the original number per group. Twelve animals were in each group.

[b] Standard error of the mean.

rose, animals fed the above diets
d a highly significant retardation in
gain, lack of grooming, varying de-
f alopecia, extensive diarrhea, and
ortality within an experimental pe-
14 days. With the exception of
retardation during the first week of
, these effects, except for a slight
a, were completely counteracted
ne diet contained cornstarch as the
of dietary carbohydrate, and animals
latter diet appeared grossly normal
espects. Intermediate results were
d on a diet containing dextrin as the
carbohydrate. No data are available
he *modus operandi* whereby corn-
exerted its protective effect. It has
ggested that the activity of various
fibers in counteracting the toxicity of
doses of sodium cyclamate and
od additives may be related to their
on the kinds of organisms present
ir metabolic activity in the intestinal
). Further studies are indicated to
ne to what extent diets containing
ch and other complex carbohy-
n a natural food stock ration may
e intestinal flora in contrast to find-
tained with diets containing sucrose
ose as the source of dietary carbohy-

able data indicate that the protective
of dextrin and cornstarch in contrast
se and dextrose as sources of dietary
drate in rats fed a purified, low-fiber
not confined to rats fed toxic doses
m cyclamate. In preliminary studies
ed in this laboratory,[2] with six rats
up on diets identical to those em-
above, except that FD & C Red No.
anth) replaced sodium cyclamate in
, all rats fed the sucrose- or dextrose-
ing diets succumbed within an exper-
period of 14 days with a maximum
increment during this period of 6 g.
rast, four out of six rats on both the
and cornstarch-containing diets sur-
he experimental period of 14 days
average weight increment of 41 and
spectively. The latter weight incre-
as considerably less than that ob-
vith various dietary fiber-containing

materials when incorporated in a purified, low-fiber diet which contained 5% FD & C Red No. 2 and sucrose as the source of dietary carbohydrate (4). It was also significantly less than that obtained on a natural food stock ration supplemented with 5% FD & C Red No. 2 (4). The above findings suggest that the protective effects of a natural food stock ration in rats fed a diet containing 5% sodium cyclamate or 5% FD & C Red No. 2 were due in part to the kind of carbohydrates present in the natural food stock ration.

Present findings are in accord with the hypothesis initially proposed by Cleave (8), and subsequently elaborated upon by Cleave and associates (9, 10), that a number of disorders of Western man (including constipation, diverticular disease, irritable colon, diabetes, coronary disease, obesity, and others) is due in part to a marked change in the type of foods he eats compared to what he ingested in the past. During the past 200 years, a marked increase has occurred in Western societies in the per capita consumption of sugar. Associated with this change, due in large part to the introduction of roller milling between 1877 and 1880, a reduction occurred in the amount of dietary fiber ingested by Western man, particularly of the type present in cereal grains. Present findings indicating the marked toxicity of sodium cyclamate in rats fed a low-fiber diet containing sucrose as the source of dietary carbohydrate, and earlier findings indicating the absence of such toxicity in rats fed a similar amount of sodium cyclamate in conjunction with a natural food stock ration (which contained a number of diverse dietary fibers as well as complex polysaccharides as the prime source of dietary carbohydrate) (3), suggest that Cleave's hypothesis may also be applicable to other physiologic disturbances and to species other than man.

Summary. Immature male rats were fed a purified, low-fiber diet containing 5% sodium cyclamate but varying as to the source of dietary carbohydrate. On diets containing sucrose or dextrose as the dietary carbohydrate, animals exhibited a highly significant retardation in weight gain, lack of grooming, varying degrees of alopecia, extensive diarrhea, and high mortality within an experimental period of 14 days. With the ex-

Ershoff, unpublished data.

ception of growth retardation during the first week of feeding, these effects except for a slight diarrhea were completely counteracted when the diet contained cornstarch as the source of dietary carbohydrate, and animals appeared grossly normal in all respects and comparable to those fed a purified, low-fiber, cyclamate-free diet. Intermediate results were obtained in rats fed sodium cyclamate in conjunction with a diet containing dextrin as the source of dietary carbohydrate.

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Received June 4, 1976. P.S.E.B.M. 1977, Vol. 154.

INGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 69-71 (1977)

ence for a Free-Running Pituitary-Adrenal Circadian Rhythm in Constant Light-Treated Adult Rats (39606)

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the rat, the zenith of the plasma corti-
rone circadian cycle can be shifted by
ing lighting conditions (1, 2), suggest-
hat light-dark cycles entrain the circa-
rhythm of the pituitary-adrenal sys-
The effect of constant light on this
ım has not been established. Group
based on samples from different rats at
time point, demonstrated loss of
ımicity in rats in a constant light envi-
ıent (2-4). However, group data can-
listinguish between loss of synchroniza-
of rhythms and loss of rhythmicity. To
: this distinction it is necessary to exam-
erial samples from individual rats. Us-
ıch samples, Krieger (4) found that 80-
)ld rats reared in constant light from
did not have a circadian rhythm in
ıa corticosterone.

determine whether constant light also
shes the circadian rhythm in individual
rats, we studied female rats placed in
:ant light as adults. These rats were
ted from those in an experiment de-
d to determine the temporal sequence
interrelation of the disappearance of
us cycles and AM-PM variations in
ıa TSH and corticosterone in constant
(5). At the time of the present study,
ınimals were in a state of persistent
ıal estrus and had lost the normal nyc-
neral pattern of plasma TSH and corti-
rone as defined by two sample collec-
per day.

ıterials and methods. Adult female
gue-Dawley rats (240-260 g, Simon-
received when approximately 11 weeks
were maintained under conditions of
'olled fluorescent lighting (lights on
-1800 hr) and temperature (24 ± 1°).
ıa lab chow and water were available at
mes. After 2 weeks of acclimation to
aboratory conditions, 19 of the 29 rats
moved to an adjacent room of similar
size and shape in which the lights were constantly on. Light intensity was between 10 and 20 fc. Vaginal smears, taken 5 days per week, were used as an index of estrous cycles. As reported separately (5), AM and PM blood samples were collected at various times before and after the rats were placed in constant light to determine how quickly normal AM-PM variations in TSH and corticosterone were lost. After 15 weeks in constant light, nine rats which demonstrated persistent estrus were selected for serial blood sampling. Nine of the rats which had been maintained on the usual light-dark schedule and had normal estrous and TSH cycles were used as controls.

Blood samples were taken from a tail vein at 4-hr intervals over a 44-hr period. The validity of this technique for demonstrating normal variations in concentrations of plasma corticosterone has been demonstrated (4, 6) and the method used in this experiment has been described (6). In brief, 0.3 ml of blood was obtained from a 2- to 5-mm incision over a tail vein within 3 min after removing a rat from its cage. Rats were housed in individual cages 3 days prior to and during the experiments. The bleeding order was arranged to alternate between constant light (LL) and light dark (LD) maintained rats.

Plasma corticosterone was measured with a semiautomated microfluorescence technique (7). Statistical probabilities were determined by one-way or two-way analysis of variance for repeated measures (ANOVA) (8). Statistical evaluation of corticosterone patterns in individual rats was difficult because of the small number of data points. In addition, corticosterone concentrations varied with time in the form of sharp peaks rather than as a sine wave making sinusoidal regression analysis inappropriate. The following criteria were empirically derived and

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used to determine whether an individual rat had a corticosterone (B) circadian rhythm: (i) The highest plasma B value on both days occurred at the same time of day plus or minus 4 hr. (ii) There was a positive correlation coefficient correlating plasma B values on both days with time. (iii) The correlation coefficient was within 2 SD of the mean of the control LD group. (iv) The ratio of the maximum to minimum plasma B was within 2 SD of the mean of the ratio of the control group on both days. Any rat which did not meet all four criteria was considered not to have a circadian rhythm. Application of these criteria gave results which agreed with subjective judgments based on inspection of graphed data. The one control rat which was arrhythmic was not used in calculating mean and SD for the control data.

Results. Five of nine LL rats and eight of nine LD rats had fluctuations in plasma corticosterone with a periodicity of approximately 24 hr. The eight rhythmic LD rats had high plasma corticosterone concentrations at approximately the period when the lights went off on both days. Steroid peaks in LL rats were not synchronized with time of day or with those of other rats in the group (one at 0600, one at 1000, two at 1800, one at 2200). Arrhythmic fluctuations were observed in four LL and one LD rat. Corticosterone patterns of representative rats are shown in Fig. 1.

Figure 2 illustrates that the pattern of the LD group obtained by this sampling technique was consistent with normal, synchronized 24-hr periodicity. Plasma corticosterone in the LD group showed variations with time ($P < 0.01$, one-way ANOVA) and the mean peak occurred at 1800 on both days. The LL group did not demonstrate a rhythmic pattern and fluctuations were not significant during the sampling period ($P > 0.05$, one-way ANOVA).

Discussion. The presence of an asynchronous 24-hr periodicity in plasma corticosterone concentration in five of the LL rats indicates that these rats had an unentrained, "free-running," circadian rhythm in pituitary-adrenal function. The other four LL rats had aperiodic corticosterone fluctuations suggesting loss of rhythmicity. This study illustrates the importance of obtaining serial samples from individual rats to distinguish between loss of rhythmicity and loss of synchronization. The arrhythmicity in four of nine LL rats suggests that this treatment has a disruptive effect on the corticosterone circadian rhythm. The rhythm may be grad-

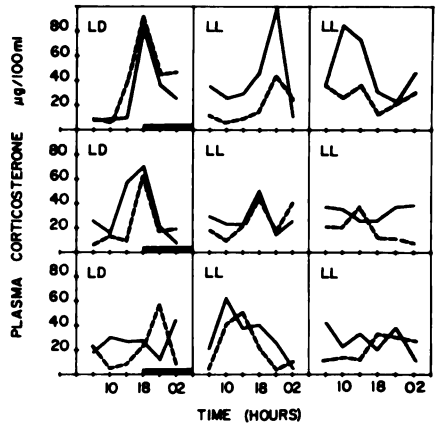

FIG. 1. Individual plasma corticosterone cycles of representative animals kept in a light–dark (LD) or constant-light (LL) environment for 15 weeks. The solid lines connect values on the first day; dashed lines connect values on the second day. The solid bars represent the dark periods. Patterns of three of the four arrhythmic LL rats are shown in the right panel, that of the arrhythmic LD rat is shown in the lower left section.

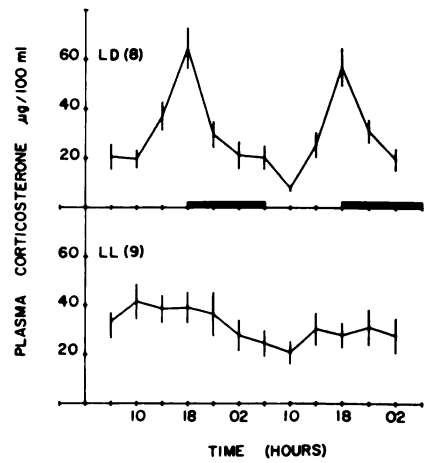

FIG. 2. Effect of constant light (LL) for 15 weeks on the group pattern of plasma corticosterone. Lines connect group means of the plasma corticosterone concentrations obtained from serial samples every 4 hr for 44 hr. Vertical lines indicate SE. The number of rats in each group is in parentheses. Dark periods for the light–dark group are indicated by the solid horizontal bars on the abscissa.

ing after 15 weeks without light-
s, or constant light may make the
nore vulnerable to the stressful ef-
he bleeding procedure. Five of the
rats demonstrated a circadian
l rhythm, indicating that constant
es not necessarily abolish this
All of the rats showed normal AM-
ences in plasma B when they were
to our standard light-dark environ-
. Similar results were obtained by
al. (9) in rats returned to a light-
ronment after 90 days of exposure
nt illumination.

oup data from LL rats in this study
ar to those obtained in other labo-
/hen different rats were sampled at
e point (2–4). There is one report
up data from constant light-treated
ts (1), indicating 24-hr corticoster-
odicity with a phase shift. The pat-
iat study may have been obtained
e or may have reflected synchroni-
an environmental cue other than

dividual patterns of five of the LL
he present study differ from the
ic fluctuations found by Krieger in
d rats reared from birth in con-
t. The discrepancy between these
uggests that the age at which a rat
in constant light is critical. A circa-
odicity in corticosterone does not
ntil 20 to 30 days of age (4) and a
othalamic tract appears at the
e as the onset of diurnal variations
sterone (10). Therefore, it is possi-
light–dark cycles are necessary to
ircadian rhythmicity in the brain–
adrenal system.

ata indicate that light–dark cycles
ne circadian rhythm of corticoster-
that they are not essential for
ty in adult rats. This interpretation
le of light–dark cycles in synchro-
uitary–adrenal rhythmicity is con-
h previous data obtained from rats
s adults which showed a free-run-
icosterone circadian rhythm 10
er blinding (11).

ry. To determine whether the cir-
cadian rhythm in pituitary–adrenal function persists in adult rats exposed to constant light, 24-hr patterns of plasma corticosterone concentrations were compared in individual constant light (LL) and light–dark (LD) (lights on 0600–18000 hr) maintained adult female rats. Nonstress plasma corticosterone concentrations were determined in serial blood samples obtained from a tail vein of each rat at 4-hr intervals for a 44-hr period 15 weeks after initiation of constant light treatment. Plasma corticosterone of eight of nine rats kept in LD had a 24-hr periodicity with peaks entrained to the onset of dark. Five of the nine LL rats demonstrated an unentrained circadian periodicity in plasma corticosterone. The other four LL rats had apparently arrhythmic fluctuations. These findings suggest that in adult rats the plasma corticosterone rhythm is not dependent on an LD environment but that constant light does have a disruptive effect on the rhythmicity of this hormone system.

We wish to thank Shirley Arneson, Patricia Panton, and Susan Jones for their excellent technical and secretarial assistance.

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Received July 29, 1976. P.S.E.B.M. 1977, Vol. 154.

The Effect of Thermal Trauma in Mice c

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Burn trauma produces an impairment of cellular immunity manifested by a prolongation of skin graft survival time, by a decrease in the delayed hypersensitivity response, and by a diminished graft versus host reaction (1–3). Since lymphocytes are important mediators of the cellular immune response, a study of their functions could elucidate the mechanisms of this phenomenon. Previous investigations on the effect of thermal trauma on several of the many functions of lymphocytes have shown a defect in the proliferative response of lymphocytes in the mixed lymphocyte culture reaction (4, 5) but no defect in their response to mitogenic stimulation by phytohemagglutinin (6, 7).

In this communication, we studied the effect of thermal injury on the cytotoxicity of lymphocytes, a property which correlates with the survival time of skin allografts.

Materials and methods. Inbred, adult, female BALB/c mice (h-2^d) and male C57BL/6N mice (H-2^b) were used as host and donor, respectively. The murine ascites tumor, EL-4, carried in adult C57BL/6N mice by passage once a week, was used as immunizing material and target cells. Mice were sensitized by a single ip injection of 3×10^7 tumor cells in 0.1 ml of phosphate-buffered balanced salt solution (BSS). Lymphoid cells from spleen and mesenteric lymph nodes were harvested as previously described (8). Spleens from two or three mice and lymph nodes from four mice were pooled for each experiment. For each assay of lymphoid cells from peripheral blood, 10 identically treated BALB/c mice were decapitated and bled into 0.5 ml of heparin. The blood was diluted with an equal volume of BSS, and the lymphocytes were separated by a Ficoll-Hypaque gradient.

For the cytotoxic assay, previously described methods (8, 9) with some modifications were used. One milliliter of attacking

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hetized BALB/c mice were given a ds body surface area burn in water r 7 sec and were treated with 3 ml of NaCl sc. The anesthesia produced stically significant effect on cytotox- lymphocytes.

preincubation experiments (Table nl phosphate-buffered saline (PBS), 0.4 ml of mouse sera, or 0.1 ml of rtisone (HC: hydrocortisone sodium ate, Merck, Sharp & Dohme) were o each tube of 1 ml of attacking cells d from the spleens of normal mice after sensitization (Table I, column allowed to stand for 1/2 hr at room ture before adding 1 ml of target he cytotoxic assay was then carried Table I, column B, the same quanti- PBS, serum, or HC were added to e of attacking cells and incubated at 1 hr with shaking. Afterwards, the re washed two times with 1 ml of medium. Then the attacking cells constituted with 1 ml of Eagle's me- fore addition of 1 ml of target cells, l by the cytotoxic assay. In Table I, C, the same procedure was used ml of target cells as described for attacking cells in B, but in the last step, 1 ml of attacking cells was added after reconstitution of target cells.

For statistical analysis, the probability of 0.05 ($P = 0.05$) was chosen as the level of statistical significance. Statistical differences between groups were calculated with the Student's t test. The data in Figs. 2 and 3 were plotted as a ratio of burned:normal for the sake of clarity and brevity, and statistical significance for the difference between the means of the quadruplicate samples of burned and unburned mice was calculated for each point of the figure.

Results. The effect of thermal trauma on cytotoxicity of sensitized lymphocytes. Preliminary experiments on normal BALB/c mice sensitized with EL-4 tumor cells showed a linear relationship between ^{51}Cr release from target cells and attacking cells at a concentration of 5×10^5 cells per tube for target cells and at a concentration of 2, 1, and 0.5×10^6 cells per tube for spleen and peripheral blood lymphocytes, or at 4, 2, and 1×10^6 cells per tube for mesenteric lymph node lymphocytes. Figure 1 illustrates the typical development of cytotoxic cellular immunity with time in unburned

E I. Effect of Postburn Serum and Hydrocortisone on Cytotoxicity of Spleen Cells from Unburned, Sensitized Mice.

	A No preincubation at 37° (% lysis)	B Preincubation of attackers at 37° (% lysis)	C Preincubation of targets at 37° (% lysis)
ditions	Mean ± SD	Mean ± SD	Mean ± SD
	29.7 ± 0.56	23.9 ± 1.88	23.4 ± 1.59
ouse serum	36.9[a] ± 1.81	36.4[a] ± 1.15	25.2 ± 1.40
burn serum	34.5[b] ± 1.91	30.3[a] ± 1.27	26.4 ± 2.35
burn serum	31.3 ± 3.48	23.0 ± 0.50	
	49.1 ± 0.61	36.6 ± 1.63	33.9 ± 1.88
ouse serum	57.2[a] ± 0.49	45.0[a] ± 1.78	35.5 ± 0.70
stburn serum		41.6[b] ± 1.50	31.8 ± 0.92
stburn serum		41.7 ± 3.04	34.0 ± 2.90
	55.4 ± 3.1	45.1 ± 0.7	50.8 ± 1.7
μg/ml	50.5[c] ± 2.0	37.8[a] ± 1.9	49.7 ± 2.4
μg/ml	55.2 ± 1.6	41.1[a] ± 1.9	47.7 ± 1.8
3 μg/ml	52.9 ± 2.3		
5 μg/ml	57.5 ± 0.5		
05 μg/ml	58.0 ± 1.3	40.6[b] ± 2.3	49.1 ± 1.6

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cal significance was calculated on the difference between each value in the column and its corresponding ith PBS.

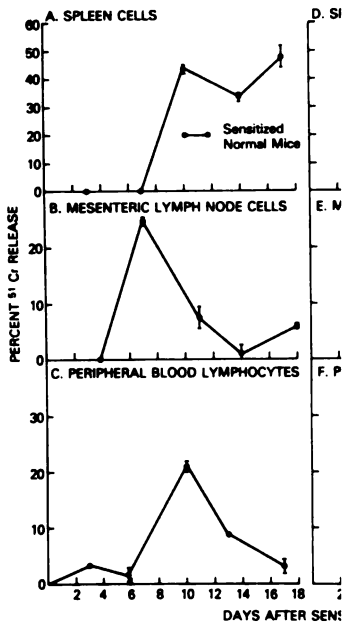
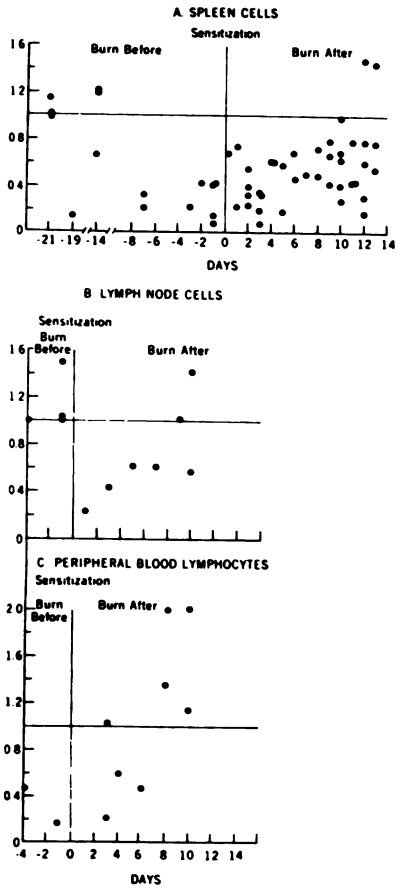


FIG. 1. The development of cytotoxic lymphocytes of B tumor cells. The cytotoxicity assay was carried out with 2 × blood, but with 4 × 10^6 attacking cells from mesenteric lymp analyses of each organ were performed on the same day, but and burned mice was not simultaneous. The burn is described mean ± SD of pooled organs, as described in Materials and M obtained when the experiment was repeated two to three tir

and burned mice. In unburned mice, there was a sharp increase in cytoxicity around 10 days after sensitization in the case of spleen and peripheral blood lymphocytes (A and C), whereas maximum cytotoxicity of mesenteric lymph node lymphocytes from these mice occurred slightly earlier, around 7 days after sensitization (B). When BALB/c mice were burned 1 day before sensitization, there was no essential change in the time required for development of peak cytotoxic activity (Fig. 1D-F). For this reason, *in vitro* studies were usually performed with lymphocytes collected at 7 or 10 days after sensitization, depending upon the source.

Figure 2 shows the effect of thermal trauma given at various times before or after sensitization on the cytotoxic cellular immune response. In Fig. 2A, the results demonstrate a significant decrease of cytotoxicity when the burn was given any time from 7 days before sensitization to 13 days after sensitization ($P < 0.05$ with all ratios <



1. The effect of thermal trauma on cytotoxic- < 10^6 lymphocytes of BALB/c mice sensitized 4 tumor cells. Each point refers to a separate nt comparing pooled samples (see Materials hods) from burned and unburned mice. The lescribed in Materials and Methods. Spleen harvested in normal and burned mice 10–11 r sensitization, except when the burn was er 9 days. In the latter case, harvesting was n both burned and unburned mice until 1–3 r thermal trauma.

ytotoxicity of peripheral blood lym- s. Significant enhancement occurred two experiments with ratios around nd 10 days ($P < 0.01$).

ffect of burn serum on cytotoxicity of d lymphocytes from normal mice. shows a typical experiment of the f postburn sera, as well as normal sera, on the cytotoxicity of spleen llected from normal, sensitized mice. In column A, both normal and 1-hr postburn sera showed enhancement of cytotoxicity ($P < 0.05$). Enhancement also occurred when these sera, in addition to 1-day postburn serum, were preincubated with attacking cells (column B). In both cases, the enhancement was less with 1-hr and 1-day postburn sera than with normal sera. Neither normal nor postburn sera showed any significant effect on preincubation of target cells with sera before the assay (column C).

The effect of hydrocortisone on cytotoxicity of sensitized lymphocytes from normal mice. Table I shows that hydrocortisone was able to reduce cytotoxicity by 9% at a concentration of 480 μg/ml when there was no preincubation at 37° ($P < 0.05$). When hydrocortisone was preincubated with attacking cells, cytotoxicity was decreased by 16% at a concentration of 480 μg/ml, but even by 10% at a concentration of only 0.05 μg/ml ($P < 0.02$). The drug had no significant effect (2–6% inhibition) on preincubation with target cells.

The effect of lymphocytes from burned animals on cytotoxicity of sensitized lymphocytes from normal mice. Table II shows the results of experiments designed to detect "suppressor" cells. When lymphocytes from the spleens of burned mice were added to the cytotoxicity assay, there was a significant inhibition of cytotoxicity by spleen cells collected on Days 1, 2, 3, 9, 12, and 16 after burning (column A compared with column C); however, the addition of spleen cells from normal mice to the assay also showed the same phenomenon (column B compared with column C). Only on 3- and 9-days postburn was the difference between cells from burned and normal mice significantly different ($P < 0.05$) (column A compared with column B). These results indicate that burn injury does not produce a significant number of "suppressor" cells in the spleen which affect function of the preformed cytotoxic cells.

Discussion. The decreased cytotoxicity of spleen and lymph node cells, as well as peripheral blood lymphocytes from burned animals, could be responsible for the prolonged survival time of skin allografts after thermal injury. The effects of burning are

TABLE II. EFFECT OF NONSENSITIZED SPLEEN CELLS FROM BURNED AND NORMAL MICE ON CYTOTOXIC OF NORMAL, SENSITIZED SPLEEN CELLS.[a]

Days postburn when spleen cells were harvested	A Normal sensitized + burn (% lysis)	B Normal sensitized + normal (% lysis)	C Normal sensitized al (% lysis)
	Mean ± SD	Mean ± SD	Mean ± SD
1	15.3[c] ± 0.76	14.6[c] ± 0.99	18.2 ± 0.33
2	27.0[d] ± 1.06	27.3 ± 1.39	29.3 ± 0.82
3	30.2[b] ± 1.62	33.3[b] ± 0.94	38.9 ± 0.65
4	9.0 ± 1.94	8.6[e] ± 0.35	10.2 ± 0.70
7	9.1 ± 1.01	8.6[e] ± 0.35	10.2 ± 0.70
9	12.8[b] ± 0.17	14.6[c] ± 0.99	18.2 ± 0.33
12	33.2[b] ± 0.48	33.3[b] ± 0.94	38.9 ± 0.65
16	26.0[c] ± 1.73	27.3 ± 1.39	29.3 ± 0.82

[a] Cytotoxicity was measured using 2×10^6 spleen cells from mice sensitized with EL-4 tumor cells and 5 > ^{51}Cr-labeled tumor cells. Additions listed were: burn, 2×10^6 spleen cells from burned nonsensitized r normal, 2×10^6 spleen cells from normal nonsensitized mice. Nonsensitized spleen cells from normal or bu mice produced an 8–11% release of ^{51}Cr in the cytotoxicity assay with 5×10^5 labeled target cells.

[b] $P < 0.001$.

[c] $P < 0.01$.

[d] $P < 0.02$.

[e] $P < 0.05$.

For the calculation of statistical significance, the mean values of columns A and B were compared witl control values of column C.

immediate and potent, at least in the case of spleen cells, since burning at 10–12 days after sensitization, when cytotoxicity has reached a peak, could significantly decrease that cytotoxicity. This was not usually the case with lymph node cells or peripheral blood lymphocytes.

An attempt was made to identify humoral factors in the blood of burned mice which could reduce cytotoxicity of lymphocytes, but no such evidence could be found either in serum or in plasma obtained from several hours to 4-days postburn. Because hormones, especially from the adrenal cortex, can affect cellular immunity (10), and because increased adrenal activity after trauma has been extensively documented (11), hydrocortisone, one important product of that activity, was tested on cytotoxicity of spleen cells. The drug inhibited cytotoxicity of lymphocytes at a concentration as low as 0.05 μg/ml when attacking cells were preincubated with the drug, a level of corticosteroid that occurs in the blood of normal and burned rats and humans (10, 12); however, since the inhibition was only 10%, it is doubtful whether cortisone plays an important part in the much greater inhibition observed in the *in vivo* experiments.

Since efforts to explain the marked reduction of cytotoxicity of lymphocytes by moral factors failed, a search was made suppressor cells in the spleens of bur mice to account for this phenomer Again no evidence was found to support mechanism. Since it is possible that an hibitor of cytotoxicity could be bound to cell membrane of lymphocytes, experim are underway to test this hypothesis.

Summary. The effect of thermal tra on cellular immunity was studied in inl mice using an *in vitro* assay for cytot lymphocytes from spleen, lymph node, peripheral blood. Burned BALB/c r sensitized with EL-4 tumor cells f C57BL/6N mice showed a significant crease in cytotoxicity of spleen, lymph n and peripheral blood lymphocytes. This pression of cytotoxicity occurred in sp lymphocytes when the burn was give early as 7 days before sensitization or as as 13 days afterwards. Similar results v obtained with peripheral blood lym cytes, but in the case of lymphocytes f lymph nodes diminished cytotoxicity observed only when the animals v burned after sensitization. No evidence found for an inhibitor of cytotoxicity in rum from burned mice or for "suppres cells in the spleen after thermal injury.

luced cytotoxicity of lymphocytes in med animals could be of critical impor- ce in the impairment of some manifesta- ns of cellular immunity after burn uma.

We wish to thank Dr. John R. Wunderlich of the ional Cancer Institute for his helpful suggestions discussion during the course of this work and Ms. an Sharrow of the National Cancer Institute for her ice on technical matters.

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Received July 7, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 78-

Altered Lethality of Murine Toxin from *Yersi*
Conditions (3

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The etiological agent of plague is *Yersinia pestis*, a gram-negative organism that contains a protein toxin having lethal activity only against rats and mice (1). The murine toxin has been thoroughly purified (2) and characterized (3), and the chemistry of its active site has been investigated (4). Nevertheless, the mechanism of action of this toxin has remained unclear. The effects of relatively crude preparations of the toxin in mice and rats have suggested that circulatory failure was the lethal event following peripheral vascular collapse (5, 6), whereas extensive studies with isolated rat heart mitochondria have led to the hypothesis that heart malfunction was the lethal event followed by circulatory failure (7). The latter hypothesis was developed from the observation that purified toxin preparations blocked the NADH-Coenzyme Q reductase activity in the terminal respiration system (8). An important observation was the ability of the purified toxin to inhibit the respiration of heart mitochondria from the rat and mouse but not that from the rabbit, dog, chimpanzee, and monkey, which are resistant species (9). However, the amounts of toxin which were required to inhibit mitochondrial respiration (0.5–2.0 mg/ml of mitochondrial suspension) were much greater than those needed to kill susceptible animals (0.5–3.0 μg/mouse, ip). This fact raised the possibility that a primary site of lethal activity of the toxin has not been identified. Therefore, we have examined the effects of highly purified preparations of the murine toxin in mice. This investigation shows that the susceptibility of mice to the toxin is related to their metabolic state and suggests that hypothermia may be a primary determinant of lethal activity. A preliminary report has been presented (10).

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served.

y the injection iv of alloxan mono- Sigma Chemical Co.) at 70 mg/kg They were challenged with toxin pt. I) or 120 hr (Expt. II) after the jection when fasting blood glucose eeded 200 mg/100 ml. (e) N^6,$O^{2'}$-yclic AMP (db-cAMP, Sigma) 0.5 se in saline was injected ip 5 min d 3 hr after toxin challenge. The ice received saline. (f) Groups of conditioned to a room tempera-, 17, 25, or 37° for 1 hr before and maintained at the respective re for the subsequent 48 hr.

rine effects in vivo. Mice were 4 hr before challenge with the e effect of the murine toxin on ne-induced increases in blood glu- plasma free fatty acids (FFA) was d 1.5 or 6 hr after challenge. At ach mouse was injected ip with 6 of epinephrine (Adrenaline chlo- e Davis and Co.). Heparinized d was collected from mice under thesia either 10 or 30 min following epinephrine injection. The blood was kept at ice temperature until all animals had been sampled. The concentration of blood glucose in each mouse was determined in duplicate according to Nelson (13) following protein precipitation to give a final dilution of 1:20. The free fatty acids in 1 ml of plasma were extracted and determined according to Dole and Meinertz (14) with palmitic acid as the standard. The plasma was obtained following centrifugation of blood pooled from four mice.

Results. Table 1 shows the ability of various treatments to modify the susceptibility of mice to ip challenge with the murine toxin. In each experiment, altered mouse susceptibility was manifested by a change in the time required for the toxin to kill the mice and by the number of mice killed. The toxin was more effective when mice were exposed to ambient temperatures of 5 or 17° than to 25°, whereas exposure to 37° completely antagonized lethal activity. At 25°, the toxin was most lethal when the Purina diet was replaced by the fat-free diet. In one

TABLE I. MODIFICATION OF THE SUSCEPTIBILITY OF MICE TO THE MURINE TOXIN.

ment	Conditions[a]	Expt. I 12[b]	Expt. I 24	Expt. I 48	Expt. II 48	Expt. III 48
	Purine chow vs. Fat-free diet	2/6[c]	2/6	2/6	0/8	0/4
		3/6	6/6	6/6	7/8	4/4
	Purina fed vs Fasting	5/6	6/6	6/6	5/5	4/5
		0/6	1/6	4/6	2/5	0/5
	Glucagon vs Untreated	1/5	2/5	3/5	2/7	
		5/5	5/5	5/5	7/7	
	Cortisone vs Untreated	0/5	2/5	3/5	1/4	
		5/5	5/5	5/5	4/4	
	Alloxan diabetes vs Untreated	1/6	3/6	3/6	4/7	
		5/6	6/6	6/6	7/8	
	Dibutryl cAMP vs Untreated	1/6	3/6	3/6	1/5	1/3
		4/6	5/6	5/6	4/5	3/3
nperature	5° vs			5/5		
	17° vs			4/5	8/8	5/5
	25° vs			2/5		2/5
	37°			0/5	0/8	

thods for details.
fter challenge with the murine toxin. Control and treated samples were injected with the same toxin ₅₀ units).
· of deaths/number of mice challenged.

experiment (Expt. III), a sublethal dose of toxin in Purina-fed mice was fully lethal in animals receiving a fat-free diet. In contrast, the lethal expression of the toxin was decreased in fasting and diabetic mice. Likewise, the injection of pharmacological amounts of glucagon (0.36 and 0.52 mg), cortisone (5 and 10 mg), or db-cAMP (1 mg) antagonized toxin lethal activity. However, the injection of pharmacological doses of epinephrine was consistently without effect in modifying the lethal activity of the toxin.

Further experiments were performed to determine the responsiveness of toxin-challenged mice to epinephrine because the physiological response to epinephrine is important in maintaining the metabolic and mechanical hemeostasis. The data in Table II show that the mobilization of free fatty acids in response to exogenous epinephrine was completely blocked by the murine toxin at 1.5 hr after challenge. Figure 1 shows that the toxin also blocked the ability of epinephrine to induce hyperglycemia in mice at 6 hr after challenge. The animals contained demonstrable liver glycogen and the toxin did not change plasma levels of immunoreactive insulin (D. Wennerstrom and T. Montie, unpublished). Neither response to epinephrine was affected by toxin that had been inactivated by heat (toxoid).

TABLE II. THE EFFECT OF THE MURINE TOXIN ON EPINEPHRINE-STIMULATED MOBILIZATION OF FREE FATTY ACIDS.

Experiment	Challenge[a]	Free fatty acids (μEq/liter) +Buffer	Free fatty acids (μEq/liter) +Epinephrine
I	Buffer	1184 ± 15[b]	1763 ± 15
	Toxoid	1210 ± 21	1647 ± 33
	Toxin	986 ± 13	1026 ± 15
II	Buffer	1212 ± 17	1909 ± 25
	Toxoid	1181 ± 25	1742 ± 15
	Toxin	1015 ± 15	1075 ± 15

[a] The mice were fasted for 4 hr prior to ip challenge with buffer, toxoid, or toxin (1 μg). After an additional 1.5 hr, each mouse was injected ip with buffer or buffer containing 10 μg of epinephrine. Heart blood was collected 10 min later.

[b] Each value represents the mean ± the standard error for duplicate determinations on duplicate pools of plasma from four mice.

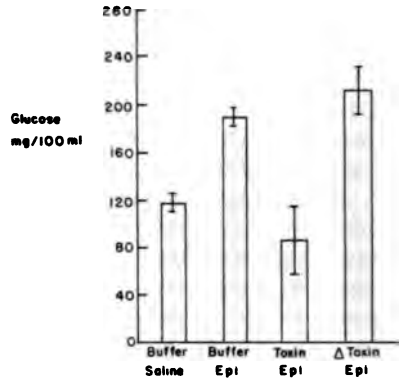

FIG. 1. The effect of toxin on epinephrine-induced hyperglycemia in fasting mice. Mice were fasted 4 hr prior to challenge ip with 0.3 ml of 10 m*M* Tris-chloride buffer (pH 7.6), 3 μg of toxin A in 0.3 ml of the buffer, or 3 μg of toxin A heat-inactivated (Δ toxin) at 70° for 30 min in the buffer. After 6 hr, each mouse was injected ip with saline or 6 μg of epinephrine (Epi) and bled after 30 min. Each bar is the mean value of individual samples determined in duplicate ± the standard error for five mice per group, except the toxin group which is from four mice.

Discussion. The mechanism of action of *Y. pestis* murine toxin *in vivo* has been controversial (15). A previous investigation has shown that crude preparations induced pooling of blood and hypothermia in rats and mice (5). The vascular system was not affected by exogenous epinephrine and the loss of blood pressure was confirmed in rats challenged with partially purified toxin preparations (6). These studies suggested a direct action of the toxin on peripheral vascular elements. In contrast, our results which show that the effectiveness of highly purified murine toxin could be modified by the nutritional and hormonal state of the challenged mice and by the ambient temperature, suggest that disruption of normal metabolism and the consequent development of hypothermia are primary determinants of lethal activity. The impairment of metabolism by the toxin is supported by the inability of challenged mice to mobilize FFA and glucose in response to exogenous epinephrine. This finding is consistent with the negative effects of this hormone in modifying toxin lethality noted previously (5), and further suggests that the toxin impaired the metabolic and calorigenic activity controlled

by the sympathetic nervous system. Because sympathetic amines increase the oxygen consumption of myocardium (16), it might be argued that the ineffectiveness of epinephrine favors the hypothesis of a direct action of the toxin on the electron transport chain of heart mitochondria (7). However, the decreased susceptibility of mice in the hypermetabolic state (increased oxygen consumption) of diabetes (17) and the increased susceptibility induced by the hypermetabolic state of the high carbohydrate fat-free diet (18) suggest that the toxin interfered with metabolism at a level other than respiration. Our results suggest that susceptibility to the toxin is related to the mobilization and metabolic use of lipid because FFA is preferentially oxidized in fasted or diabetic mice whereas glucose is preferentially oxidized in mice receiving a high carbohydrate fat-free diet (19). Additionally, the metabolic use of lipid is promoted by glucagon and cyclic AMP and by cortisone which enhances the activity of glucagon (20).

The high susceptibility of animals exposed to the cold can be attributed to an increased blockage of FFA mobilization and resulting hypothermia (S. Brown, Ph.D. dissertation, University of Tenn., manuscript in preparation). Mice placed at 37° showed no alteration in epinephrine-induced mobilization of FFA. Thus, the increased resistance of animals placed at 37° is correlated with the decreased effect of toxin in inhibiting epinephrine-induced mobilization of FFA.

Summary. The susceptibility of mice to highly purified preparations of the murine toxin of *Y. pestis* was decreased significantly when the animals were fasted, or made diabetic, or exposed to an ambient temperature of 37°, or injected with glucagon, dibutryl cyclic AMP, or cortisone. In contrast, the lethality of toxin was significantly increased in mice that received a high carbohydrate fat-free diet and that were exposed to temperatures of 5 or 17°. In contrast to controls, epinephrine was unable to elicit hyperglycemia or increase the plasma level of free fatty acid in animals challenged with the toxin. These results support a mode of action *in vivo* based on hypothermia as a primary determinant of lethality.

David E. Wennerstrom, a predoctoral trainee, was supported by Public Health Service Grant No. TO 1-AI00435 from the National Institute of Allergy and Infectious Disease.

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Received June 16, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 82-85 (1977)

Positive Feedback by Estrogen and Progesterone on LH Release in Old and Young Rats[1] (39609)

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A number of studies has indicated that the hypothalamopituitary system is less responsive to stimuli that normally release pituitary LH in old female rats that have ceased to cycle than in young cycling rats (1-3). This includes the response to electrical stimulation of the hypothalamus (4), to castration (5), to ether stress (6), and to LRH administration (7). We have reported that injections of progesterone can induce ovulation and reinitiate regular estrous cycles in old constant-estrous rats (8). Progesterone also was shown to induce release of LH within 4-7 hr in ovariectomized, estrogen-primed mature rats (8, 10), and is believed to participate with estrogen in producing the LH surge on the afternoon of proestrus in the cycling rat (11, 12). It was of interest, therefore, to compare the ability of the hypothalamopituitary system of young cycling rats and old rats showing irregular cycling or constant estrus (no ovulation) to respond to the positive feedback of estrogen followed by progesterone on LH release.

Materials and methods. Animals. One-hundred-and-fifty 8- to 9-month-old female Long-Evans rats were purchased from Blue Spruce Farms, Altamont, N.Y. The rats were housed eight per cage in a temperature-controlled (25 ± 1°) and artificially illuminated (14 hr daily) animal room, and were fed a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and tap water *ad libitum*. Daily vaginal smears were taken from each of the rats for 1.5 months before they were selected for experimentation. At 9 to 10 months of age, many of the rats began to show irregular estrous cycles (IRC) or constant estrus (CE). A group of IRC rats and a group of CE rats were unilaterally ovariectomized (OVX) at 10 to 11 months of age, in order to first determine the effect on compensatory hypertrophy of the remaining ovary. Ten days later, the other ovary was removed. For comparison, a group of normal-cycling female rats of the same strain were similarly OVX at 60 to 70 days of age. All OVX rats were given a single im injection of 0.2 ml of Bicillin (Wyeth Labs., Inc., Philadelphia, Penn.) postoperatively to prevent infection.

Experiment 1. At 25 days after both ovaries were removed, each rat was given a single sc injection of 8 μg of estradiol benzoate (EB) in 0.1 ml of corn oil per 100 g of body weight. Seventy-two hours later, each rat was given a single sc injection of 0.8 mg of progesterone in 0.1 ml of corn oil per 100 g of body weight. A blood sample of 1.0 ml was collected by ocular sinus puncture under light ether anesthesia immediately prior to administration of progesterone at 1100, 1500, 1700, 1900, and 2100 hr.

Experiment 2. At 52 days after removal of both ovaries, the young IRC and CE rats again were given EB, followed 72 hr later by progesterone. Sequential blood samples of 0.5-ml each were taken at 1100, 1500, 1700, and 1900 hr by cardiac puncture under light ether anesthesia. The injection of progesterone was given at 100 hr.

LH radioimmunoassay (RIA). Blood

[1] Supported in part by NIH Research Grants No. AG 00416 from the National Institute on Aging, and No. AM 04784 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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s were stored in a refrigerator at 4° ght before centrifuging at 4000*g* for ı in a Sorvall refrigerated centrifuge. rum was stored in a 2-ml serum vial, l with parafilm, and kept frozen at ıntil assayed. LH concentration in in- ıl serum samples were determined by ble-antibody RIA using an ovine- system (13). Ovine LH antiserum and purified ovine LH for radioiodi- were generously provided by Dr. G. wender of Colorado State University, ollins, Colo., and by Dr. L. E. Reich- , of Emory University, Atlanta, Ga., ively. Concentrations of LH in the were expressed in terms of the refer- tandard, NIAMDD rat LH-RP-1. lifferences between groups of $P <$ ere considered to be significant.

lts. Pituitary LH response to estrogen ogesterone 28 days after OVX. At 72 r a single injection of EB, serum trations of LH were 155 ± 25, 111 ± 1 123 ± 22 ng/ml in the OVX young, rmer IRC and CE rats, respectively , left panel). After administration of terone at 1100 hr, serum concentra- f LH in the young rats rose to 428 ± ml at 1500 hr and reached a peak of ± 234 ng/ml at 1700 hr. Thereafter, LH declined to 489 ± 94 and to 139 ıg/ml at 1900 and 2100 hr, respec- In contrast, the former IRC rats l only a small rise in serum LH in se to administration of progesterone, LH peak of 852 ± 190 ng/ml at 1700 e previous CE rats showed a rise of LH similar to that of the young rats, ıg 1618 ± 320 ng/ml by 1700 hr. average body weights of the OVX and former IRC and CE rats were 5, 355 ± 9, and 365 ± 11 g, respec- Ten days after unilateral OVX, the and IRC rats showed significantly mpensatory hypertrophy of the re- g ovary than in the young rats ($P <$ Since the doses of EB and progester- re given on a per 100-g body-weight he young rats received somewhat less l progesterone than the old rats.

tary LH response to estrogen and pro- ne 55 days after OVX. At the time EB and progesterone were adminis- tered, the average body weights of the OVX young, and former IRC and CE rats were 341 ± 9, 379 ± 11, and 369 ± 9 g, respectively. The results in Fig. 1, right panel, show the patterns of serum LH responses. It can be seen that 72 hr after a single injection of EB, serum concentrations of LH were 132 ± 15 and 107 ± 24 ng/ml in the OVX young and former CE rats, respectively, whereas serum LH was only 68 ± 7 ng/ml in the former IRC rats. After injection of progesterone at 1100 hr, serum concentrations of LH in the young rats increased to 493 ± 72 ng/ml at 1500 hr, reached a peak of 2870 ± 750 ng/ml at 1700 hr, and declined thereafter to 1334 ± 598 ng/ml at 1900 hr. The previous IRG rats showed only a small LH response to progesterone increasing from 132 ± 27 to 521 ± 162 ng/ml by 1700 hr and to 647 ± 140 ng/ml by 1900 hr. Administration of progesterone induced an intermediate surge in serum LH in the previously CE rats, which increased from 221 ± 47 to 1141 ± 242 ng/ml at 1700 hr and to 1335 ± 283 ng/ml at 1900 hr. Serum LH in the old female rats rose continuously whereas in the young rats it reached a peak by 1700 hr.

Discussion. The present study demonstrates that after OVX, estrogen-primed young and old rats exhibited distinctly different patterns in release of LH 4 to 8 hr after progesterone administration. The surge in serum LH was markedly decreased in old IRC rats 28 days after OVX, and in both IRC and CE rats 55 days after OVX, as compared to that of young rats. Since we previously observed that old female rats showed a significantly smaller rise in pituitary LH release after ovariectomy than young rats (5), this together with the present results indicates that the ability to secrete LH in aging female rats is diminished both after removal of negative, or by positive feedback of ovarian hormones.

The results from the second experiment demonstrate that in the old OVX former IRC and CE rats, the LH rise after steroid treatment not only was smaller than in young rats, but also was delayed and/or prolonged. This is believed to constitute additional evidence that the LH release mechanism in old female rats is more sluggish than in young rats. This also is indicated in the

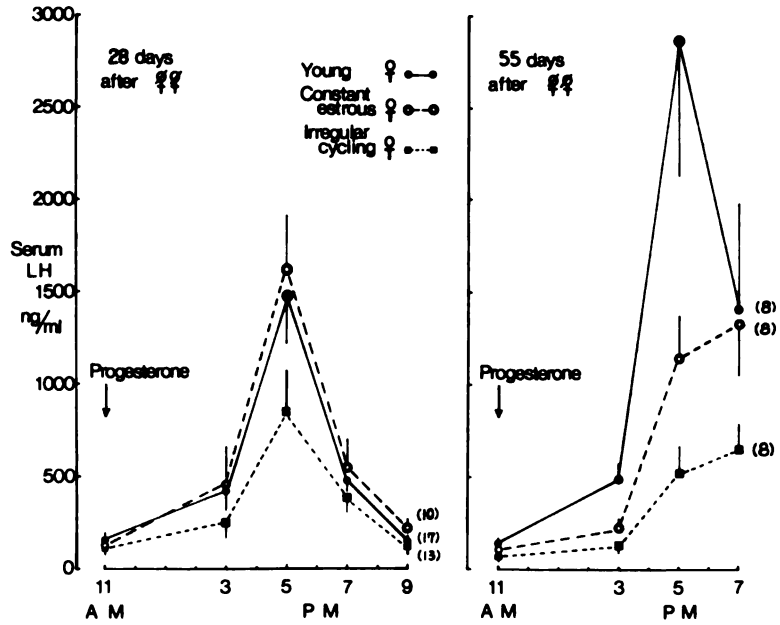

FIG. 1. Serum concentration of LH 25 or 55 days after ovariectomy (♀♀) and treatment with a single dose of estrogen (EB) followed 3 days later with a single dose of progesterone in young and old female rats. Prior to ovariectomy, the young rats showed regular estrous cycles and the old rats exhibited constant-estrus (CE) or irregular cycles (IRC). (*n*) = Number of rats per group.

significantly smaller compensatory hypertrophy of the remaining ovary after unilateral OVX in the old rats, in agreement with a previous report by Aschheim (14).

It is of interest that the OVX former CE rats showed a greater LH response to the steroid treatment than the former IRC rats, both at 28 and 55 days after OVX. It is possible that estrogen secreted by the old CE rats sensitized the hypothalamopituitary system to subsequent estrogen-progesterone stimulation. Aging CE rats have well-developed follicles in the ovaries and show persistent vaginal cornification, suggesting unhindered estrogen action, whereas IRC rats presumably are secreting progesterone as well as estrogen, perhaps thereby rendering the hypothalamopituitary system less sensitive to positive feedback by steroid hormones. It is remarkable that these differences between the two groups of old rats persisted so long after OVX.

The present results suggest that the reduced LH response to the gonadal steroids in the old as compared to the young OVX rats does not depend entirely on the previous ovarian influence. We have observed similar differences in the LH response to castration alone in young and old male and female rats (5). We have suggested that a fundamental change in the hypothalamopituitary system occurs in aging rats, and believe this is associated at least in part with alterations in hypothalamic monoaminergic activity (1, 15). Preliminary evidence indicates that in old rats, hypothalamic catecholamines are reduced and serotonin is increased (16). Catecholamines, particularly norepinephrine, are believed to stimulate and serotonin to inhibit gonadotropin release in rats (16). In addition, pituitary LH release in response to LRH stimulation was shown to be reduced in old as compared to young rats (7). Thus, several mechanisms operate to reduce gonadotropin release in response to many stimuli in old as compared to young rats.

Summary. Young cycling rats 60 to 70 days of age, and old irregular-cycling or constant-estrous rats 10 to 11 months of age were ovariectomized. At 25 or 52 days after ovariectomy, they were each given a single

injection of 8 μg of estradiol benzoate/100 g of body weight, followed 72 hr later by a single injection of 0.8 mg of progesterone/100 g of body weight. Radioimmunoassay of serum LH from blood samples removed 4, 6, 8, and 10 hr after progesterone administration showed that LH rose significantly more in the young than in the old, former constant-estrous rats 25 days after ovariectomy, and more than in either group of old rats at 55 days after ovariectomy. These results indicate that the positive feedback of estrogen and progesterone on LH release is reduced in old as compared to young cycling female rats, and is believed to account at least in part for the irregular cycling, and failure of ovulation in the old constant-estrous rats.

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Received April 7, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 86–92 (1977)

Colony-Stimulating Activity in Cultures of Granulocytosis-Inducing Tumor[1, 2] (19610)

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Granulocytosis is sometimes associated with malignant tumors arising in other than hemopoietic tissue. A "leukemoid" syndrome including splenomegaly, extreme leukocytosis, reversal of the granulocyte-lymphocyte ratio, and a mild anemia was described by Bateman (1) as an accompaniment to certain transplantable myoepitheliomas of mice. Earlier history and current status of the field have been reviewed recently (2, 3). Mechanisms underlying the syndrome remain unexplained but in recent years attention has been directed to possible involvement of granulopoietic factors elaborated by the tumors (4–7).

We have studied a granulocytosis-inducing tumor which occurred in a Swiss albino mouse of the Brookhaven (Hale/Stoner) strain and has been carried through 38 animal passages over a period of 25 months. The tumor is a mammary adenocarcinoma, the most commonly occurring spontaneous tumor in this strain (8). Our interest has focused on hematopoietic cell kinetics in host mice, to be reported elsewhere (unpublished data), and features of cell cultures of tumor tissue and the products elaborated by these cultures.

Colony-stimulating activity (CSA) is produced in high amounts over extended time periods in these cultures, making the system especially interesting and potentially valuable because, unlike other sources of CSA, these cells have the capacity to evoke, *in vivo*, gross changes in granulopoiesis. In our view, this considerably enhances the likelihood that the culture-derived products are physiologically important regulators.

Methods. Tumor is passaged every 20–22 days in 3- to 4-month-old Brookhaven strain mice by sc inoculation of 0.5 ml of a 10% (w/v) tumor cell mince in McCoy's modified medium. Hosts in passages 1–4 received 350 rad whole body irradiation before tumor inoculation. Subsequent passages (41 to date) have been carried in unirradiated hosts inasmuch as growth characteristics and hemopoietic response were found to be no different in irradiated or nonirradiated hosts.

Cell cultures, established from either tumor mince or trypsinized tumor cell suspensions, are grown in McCoy's medium plus 10% fetal calf serum (FCS) as monolayers or in suspension in spinner medium containing 2–5% FCS. Once the cultures are established, medium is collected every 3 to 8 days and stored at −20° for later treatment and assay.

Colony-stimulating activity is assayed by the agar plate method as utilized by Robinson *et al.* (9). Sample or standard stimulus (L-cell conditioned medium) is added along with 10^5 nucleated mouse marrow cells in 1 ml of McCoy's agar to 35-mm culture dishes and incubated for 7 days at 37° in a humidified 7.5% CO_2 atmosphere. Groups of 50 or more cells are scored as colonies. Samples were assayed in three to five dishes. Inhibitor to CSA was measured in cultures supplied with standard stimulus and test sample. The decrease in colonies as compared to standard stimulus alone indicated the degree of colony inhibition by the test sample.

Preparation and assay of antibody to col-

[1] This work was supported by the U.S. Energy Research and Development Administration and by NIH Grants No. HL 15685-03 and No. RO1 15237-02.

[2] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[3] Scholar of the Leukemia Society of America, Inc.

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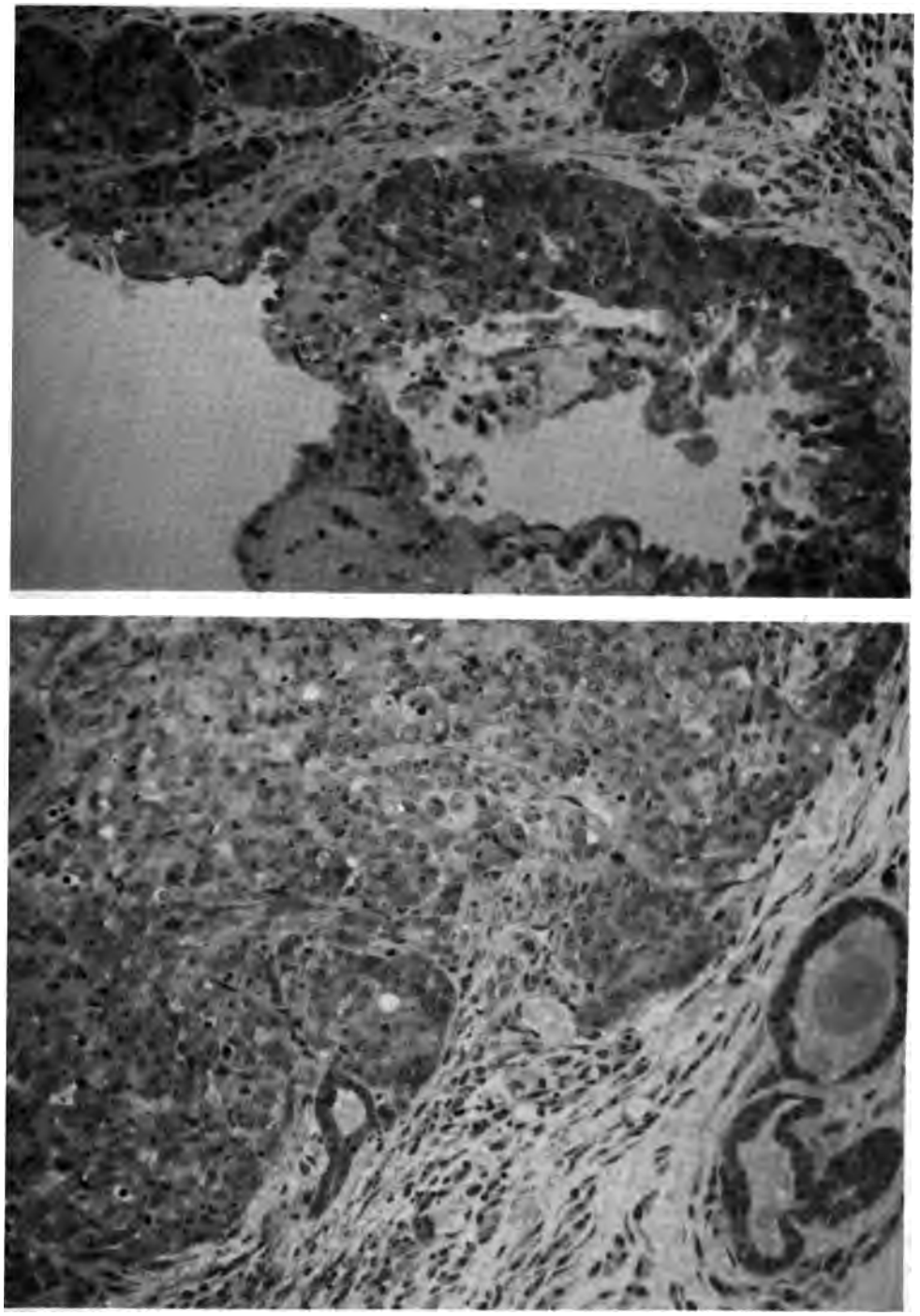

FIG. 1. Mammary adenocarcinoma; original tumor. (A) Predominantly solid components. (B) Cystic area. Hematoxylin and eosin, 140 ×.

ony-stimulating factor (CSF) was as previously described (10). Briefly, partially purified L-cell CSF was emulsified with complete Freund's adjuvant and used for immunization of rabbits. The resultant antiserum was absorbed four times with mouse red cells to remove nonspecific cross-species antibodies. The neutralizing capacity using various dilutions of 0.1 ml of antiserum was measured in the CSF assay.

Results and Discussion. Tumor characteristics. The tumor is a mammary carcinoma which has remained unchanged in morphology throughout 41 passages (Fig. 1). It bears close resemblance to the adenocarcinoma type A (11) and is composed of epithelial cells arranged in solid sheets, tubular, and cribiform structures. Some areas are cystic. The stroma is usually scarce. The core is frequently necrotic and hemorrhagic. The host may show metastatic involvement of lymph nodes and spleen. Etiology is unknown. Electron micrographs of tumor tissue failed to reveal presence of viral particles. Furthermore, ultrafiltrates of tumor homogenates did not result in tumor growth when inoculated into new-born hosts, suggesting a nonviral origin.

Subcutaneous inoculation of 0.5 ml of a 10% tumor cell mince produces palpable tumors in at least 90% of recipients in 5–7 days. By 10–14 days, the diameter is 10–20 mm. Recipients usually succumb 21–30 days postinoculation bearing ulcerated tumors up to 50 mm in diameter. Host age or sex has no appreciable influence on tumor characteristics or the ensuing hematopoietic syndrome.

Hematopoietic response. Granulocytosis develops progressively with tumor, reaching counts greater than 100,000/mm³ in some mice (Fig. 2). Differential white cell counts show granulocytes constituting 70–80% of the total, with mainly lymphocytes making up the remainder. Banded granulocytes are also increased and no cells less mature than band forms appear in peripheral blood. Extirpation of 80% or more of tumor results in a return of white cell number toward normal but regrowth provokes return of leukocytosis (Table I). The spleen, although grossly enlarged and remarkably granulopoietic (Fig. 3), appears not to be essential to development of the granulocytosis (lished data). Anemia is also a co component of the response (Fig. 4).

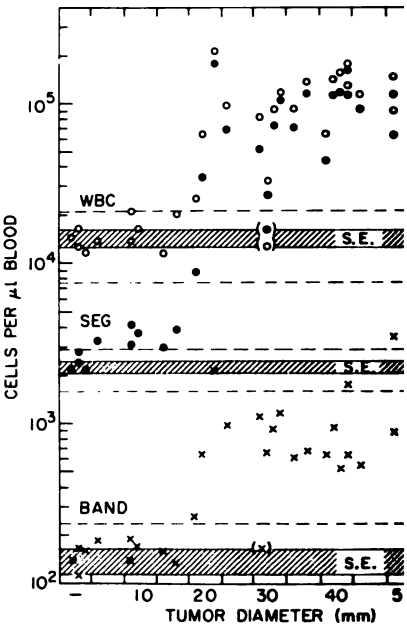

FIG. 2. Granulocytosis during tumor grow white blood cells (open circles), segmented cytes (closed circles), and banded gra (crosses) are plotted against the largest tumo ter. The graph contains data of three experime sages 4, 13, 17). Each tumor mouse is rep once. The pooled values of medium-injected mice are shown as shaded bands (mean ± error) and hatched lines (mean ± standard de

TABLE I. INFLUENCE OF TUMOR REMOVA RECURRENCE ON WHITE BLOOD COUNT

Mouse no.	Tumor diameter (mm)	Treatment	Blood co WBC
1	30	Extirpation	68,000
	20	5 Days later	32,000
2	25	Extirpation	78,800
	>20	5 Days later	30,600
	30	9 Days later	67,000
3	25	Extirpation	92,000
	–	5 Days later	18,800
	15	9 Days later	56,200

Tumor in culture. Monolayers orig from either tumor mince or trysiniz pensions consist of mixed epithelial-l

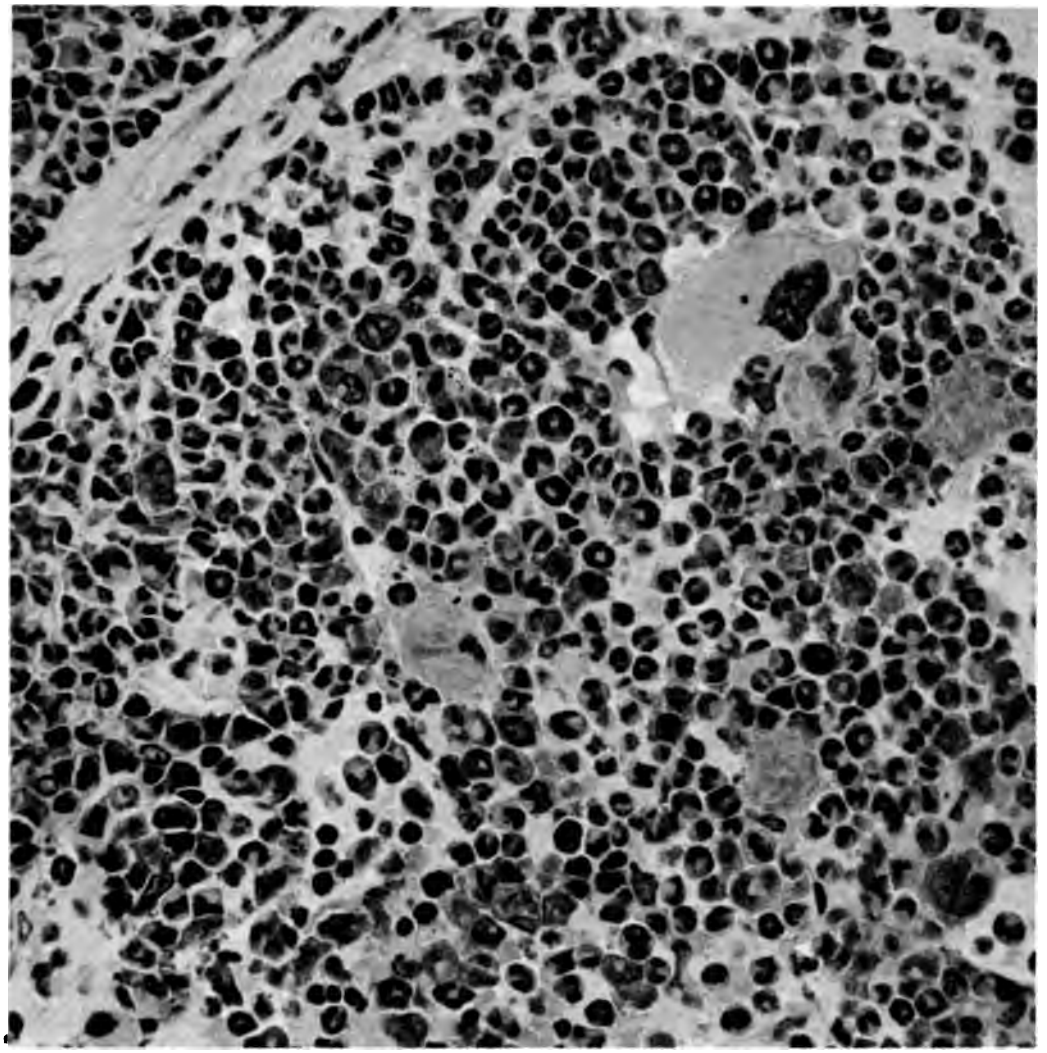

FIG. 3. Spleen of original tumor host, red pulp: granulopoiesis predominates. Small erythropoietic foci are occasionally seen (Bottom). Hematoxylin and eosin, 500 ×.

ibroblastic cells (Fig. 5). Typically the cells lisplay lack of contact inhibition, a high rate of glycolytic and protein synthetic activity, ınd a capacity for continued propagation vhen passaged repeatedly. Suspension cultures can be established from monolayers or rom fresh trypsinized tumor. They have ıeen maintained for periods up to 75 days ielding high levels of CSA at all stages of ulture.

Subcutaneous injection of the cultured ells generates mammary adenocarcinoma vith unchanged host response after multiple *n vitro* passages. Similarly, cultured cells which had been frozen and stored for 17 months displayed unaltered tumorigenicity and capacity for CSA production *in vitro*.

CSA production in culture. Both monolayer and suspension cultures consistently yield CSA to the culture medium. Activity is present in successive media collections made at 3- to 8-day intervals over periods extending to 75 days. Table II presents typical findings from two cultures. It should be noted that heat (90°, 15 min), in addition to abolishing CSA, appears to unmask or generate inhibitor activity. Media conditioned by primary cultures of mouse lung and kid-

ney and a cell line derived from rat prostatic tumor associated with leukemia had negligible CSA when assayed simultaneously as controls for mammary tumor culture media.

Characteristics of culture-derived CSA. Heat lability is a property of CSF which is presumed to be a large molecular weight glycoprotein (12). The CSA associated with tumor culture, is also inactivated by high temperature, is nondialyzable, and, from results of studies utilizing ultrafiltration membranes, the activity is associated with a fraction having a molecular weight > 30,000. This would distinguish the factor produced by our tumor cell cultures from the material studied by Delmonte and Liebelt extracted from a transplantable mouse mammary carcinoma (4). The extractable granulocytosis-producing factor was initially stated by these authors to possess physicochemical features comparable to those of erythropoietin, e.g., thermostability, pH stability, and nondialyzability. However, modification of the extraction procedure later yielded a product which the authors now characterize as a protein-free, thermostable, dialyzable (M.W. < 2000) micromolecule.

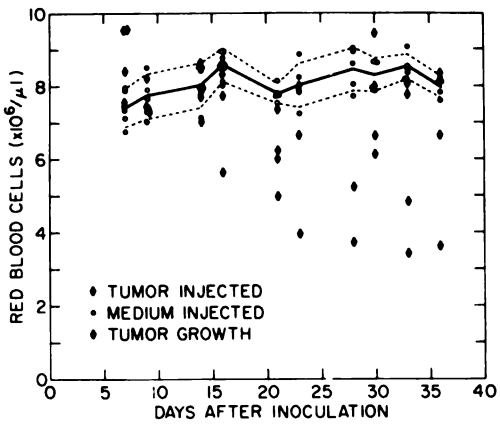

FIG. 4. Tumor-associated anemia. These data were obtained from early passages in which host mice received 350 rad whole body irradiation before tumor injection. The irradiation had no effect upon red or white cell count. It was found that preirradiation of hosts is unnecessary and unirradiated hosts have been used since passage 4.

Further characterization of the culture-derived CSA is needed before it can be equated to CSF. One strong evidence that the material is actually CSF comes from the results shown in Table III. Antibody to L-cell CSF completely neutralized the activity of tumor conditioned medium at dilutions up to 1:64. Antibody dilution (1:128), which neutralized only 20% of CSF of L-cell-conditioned medium, still abolished 88 and 72% of activity in tumor culture-condi-

TABLE II. COLONY-STIMULATING ACTIVITY IN MEDIA OF TUMOR CELL CULTURES.

Culture type	Culture age (days)	Collection interval (days)	Average number of colonies[a]		Inhibitor activity[b] (%)	
			Untreated[c]	90°, 15 min	Before 90°	After 90°
Suspension culture of mouse mammary tumor	25	3	64.7	0	11	n.d.
	33	8	72.3	0	19	n.d.
	53	7	95.3	0	5	34
Monolayer culture of mouse mammary tumor	26	5	149.3	0	0	35
	59	7	59.3	0	0	25
	75	2	36.3	0	37	n.d.
Monolayer culture of rat prostatic tumor cells (control)[d]	20	6	20.3	0	18	n.d.
	39	3	15.7	0	17	n.d.
	50	3	12.7	0	7	n.d.
Standard[e] (1) 0.03 ml			138.5			

[a] Colony = group of 50 or more cells. Average of counts in three agar plates containing 0.15 ml of tumor cell-conditioned culture medium in a total volume of 1 ml. Each plate contained 10^5 normal mouse marrow cells.
[b] Activity in 0.1 ml of tumor cell culture medium which inhibits the expected response to standard stimulus.
[c] All samples dialyzed against distilled water at 4° for 72 hr.
[d] Rat prostatic tumor cells associated with a leukocytosis.
[e] L-cell-conditioned medium.

FIG. 5. Tumor-derived cells in monolayer culture (10 days). Hematoxylin and eosin, 250 ×.

ned medium, further suggesting that the erved colony-stimulating activity is prob- y CSF.

The cell culture system reported here appears to have special value in studies of regulation of granulopoiesis. By virtue of originating from a tumor which evokes massive hematopoietic perturbations in host an-

TABLE III. NEUTRALIZATION OF COLONY-STIMULATING ACTIVITY IN TUMOR-CONDITIONED MEDIUM BY ANTIBODY TO CSF.[a]

Antibody dilution	Mixture: 0.1 ml of antibody to CSF, plus: 0.05 ml of L-cell-conditioned medium		0.05 ml of tumor-conditioned medium	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1:2	0[a]	0	0	0
1:4	0	0	0	0
1:8	0	0	0	0
1:16	0	0	0	0
1:32	4	5	1	0
1:64	17	23	0	3.5
1:128	82	87	9.5	22
1:256	n.d.	n.d.	n.d.	60
1:512	n.d.	n.d.	n.d.	68
Medium without antibody	104	105	n.d.	76

[a] Values represent the average number of colonies in three to five culture plates.

imals the culture-generated activity is more apt to be specifically related to physiologically important *in vivo* regulators of granulopoiesis than are other culture-derived factors. However, direct evidence awaits availability of enough purified material for testing in animals.

Summary. A transplantable mouse mammary carcinoma-producing granulocytosis in host mice has been established in cell cultures. Colony-stimulating activity (CSA) is released to the medium by both monolayers and suspension cultures over sustained periods. The CSA is heat labile and nondialyzable with an apparent molecular weight > 30,000. It is neutralized by antibody to colony-stimulating factor (CSF), suggesting that the material produced in tumor cell cultures is in fact CSF.

The authors are grateful for vital contributions made to this work by our collaborators, Betty Heldman, Michael Makar, Mildred Pavelec, Naomi Pappas, and Marie Susa.

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Received July 12, 1976. P.S.E.B.M. 1977, Vol. 154.

IE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 93-97 (1977)

Reduction of Exercise Dilation by Theophylline[1] (39611)

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studies, utilizing bioassay and :chniques, suggest that adenosine nd adenosine monophosphate ticipate in the genesis of exercise (1-5). Other studies show that ıe (Theo) competitively inhibits ary vasodilation produced by the adenine nucleotides (6) and o blocks the renal vascular re- ADO and AMP (7; unpublished ı). It occurred to us that Theo ;ed as a tool to further investigate le roles of ADO and AMP in 'peremia. High concentrations of ot be used in the heart because ımplicated by changes in rate and y. This is not the case in skeletal ıus, this line of approach seemed in this tissue.

. The right gracilis muscle of ɔgs was exposed and freed from tissue. All blood vessels commu- th the gracilis, except the major vein, were ligated (Fig. 1). The insertion of the muscle were tied ligatures to further ensure that ed only through the major artery ly via the major vein. The gracilis evered and distally fixed on stim- :trodes. The muscle was perfused flow by diverting femoral artery ıgh a pulsatile pump into the gra- Flow was adjusted to a level that perfusion pressure similar to sys- ial pressure and averaged 9.5 ml/ sion pressure and aortic pressure ıuously measured with low vol- re transducers coupled to oscillo- rders (paper speed 0.25 mm/sec).

experiments, contractile force ; blood oxygen tension were also measured. Force was measured with a transducer attached to the distal end of the muscle (severed from its insertion) with a ligature. The proximal end of the muscle was fixed in a clamp to prevent sliding. Venous oxygen tension was measured with an oxygen-sensitive probe interposed in a cannula which led gracilis venous blood to a small reservoir (the blood was pumped back into the animal via the contralateral femoral vein). Both variables were continuously recorded on the oscillograph; oxygen tension was also read intermittently from a digital meter.

The basic experimental protocol is illustrated in Fig. 2. After perfusion pressure reached a steady state, dose-response relationships were established for ADO, AMP, and adenosine triphosphate (ATP) by injecting 0.25, 0.5, 1.0, 5.0, 10.0, and 20.0 μg of each (0.1 ml in all cases) into the arterial line upstream to the pump. Acetylcholine (Ach), 0.5 μg (0.1 ml) and 1.0 μg (0.1 ml), was also injected. The gracilis muscle was then exercised by stimulating the gracilis nerve once per sec (6 V, 1.6 msec) for 30 sec and then six times per sec (6 V, 1.6 msec) for 30 sec.

A solution of Theo (180 mg/100 ml = 10^{-2} *M*) was infused upstream to the pump at 0.76 ml/min, producing a calculated blood concentration of $\simeq 10^{-3}$ *M*. This decreased resistance. Since an altered response to exercise is difficult to interpret when initial resistance is not the same, an infusion of norepinephrine (NE) was superimposed at a rate which returned resistance to the original value. The gracilis nerve was then again stimulated for 30 sec at both frequencies using the same voltage and pulse duration. When the response to high frequency stimulation had subsided, all doses of all drugs were again injected.

This basic protocol (B in Tables I and II) was modified in four ways. To provide a time control, Theo and NE administration was deleted and exercise was repeated 20 to

d in part before the 1975 Fall Meeting of ı Physiological Society (The Physiologist 5). Supported in part by a grant from the rt and Lung Institute.

' by the Society for Experimental Biology and Medicine

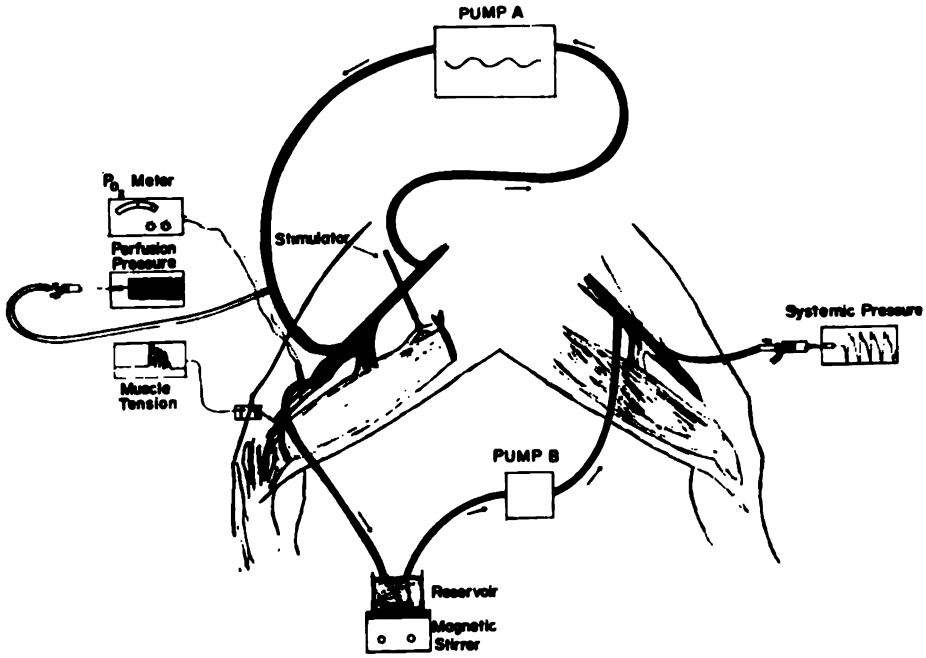

FIG. 1. Schematic drawing of the isolated gracilis muscle preparation.

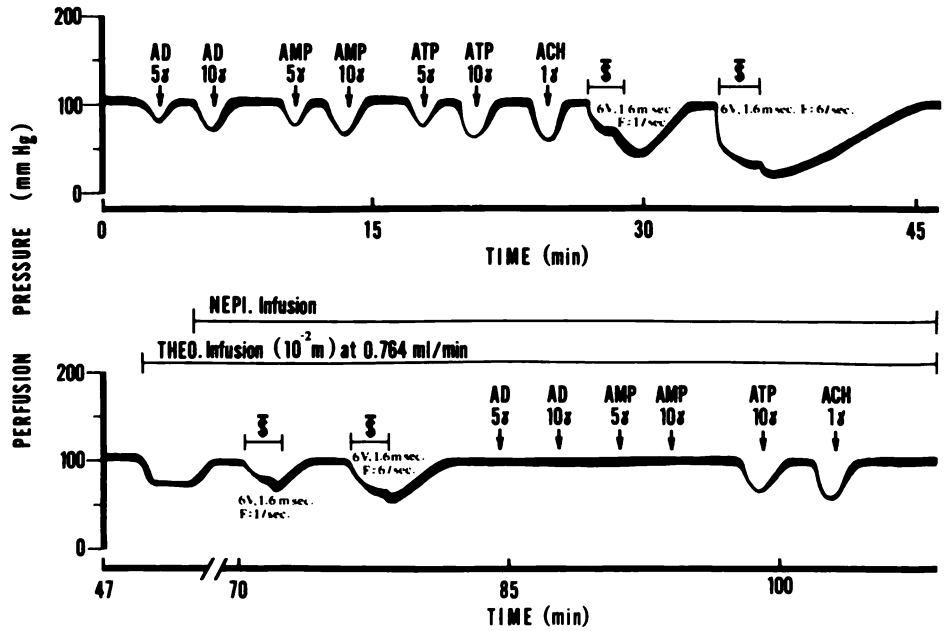

FIG. 2. Diagrammatic illustration of basic protocol.

25 min later (A in Tables I and II). To determine whether the changed exercise responses seen in series B were peculiar to NE administration, an entirely different type of vasoconstriction, vasopression (ADH), substituted for NE (D in Tables I and To determine whether the findings were culiar to the vasodilator activity of theop

TABLE I. LOW FREQUENCY EXERCISE.[a]

	Initial slope (mm Hg/sec)		Maximal decrease (mm Hg)		Response duration[f] (sec)		Area (cm²)		Muscle tension (g)	
:s	C	E	C	E	C	E	C	E	C	E
itrol	2.9	2.7	44.7	47.1	96.0	98.0	1.6	1.6	1259.0	1218.0
	±1.8	±1.9	±28.3	±29.5	±40.1	±37.9	±1.0	±0.9	±436.0	±429.0
IE	2.9	2.1	38.9	22.8	99.0	53.3	1.4	0.5	990.0	1064.0
	±1.5	±1.1**	±23.1	±12.5**	±30.0	±7.5**	±0.8	±0.1**	±570.0	±617.0
IE	1.5	0.8	17.0	9.1	78.6	48.9	0.8	0.4		
	±0.4	±0.3**	±5.2	±4.4**	±21.3	±12.0**	±0.2	±0.3**		
ADH	3.0	1.9	48.1	29.6	104.5	61.1	1.8	0.8	897.0	895.0
	±1.8	±0.7*	±20.1	±14.1**	±30.6	±17.5*	±0.8	±0.2**	±573.0	±613.0
E	2.8	3.0	48.8	46.1	106.4	109.6	1.9	2.1	1272.0	1231.0
	±1.5	±1.5	±32.7	±23.8	±43.0	±45.4	±1.4	±1.3	±401.0	±298.0

adings except the last refer to perfusion pressure. Abbreviations used: C = control period during which drugs were not administered; E = l period during which drugs were administered except in the case of series A; ' = time to 75% recovery of perfusion pressure. These 6 V, 1.6 msec, 1 pulse/sec) were used for all experiments except those in series C, where 4 V, 0.5 msec, 1 pulse/sec were used.
ant at $P < 0.05$ relative to C.
icant at $P < 0.005$ relative to C.

TABLE II. HIGH FREQUENCY EXERCISE.[a]

	Initial slope (mm Hg/sec)		Maximal decrease (mm Hg)		Response duration[f] (sec)		Area (cm²)		Muscle tension (g)	
s	C	E	C	E	C	E	C	E	C	E
rol	6.7	7.5	68.1	74.0	369.7	334.8	9.9	9.6	1190.0	1165.0
	±3.3	±4.5	±20.7	±21.3	±243.8	±175.0	±6.1	±5.7	±380.0	±379.0
E	7.4	4.8	72.8	44.2	441.6	90.1	8.0	1.3	898.0	896.0
	±4.7	±2.4*	±30.1	±18.1**	±197.9	±35.8**	±5.8	±0.6**	±490.0	±484.4
E	4.5	2.8	47.3	30.7	256.6	81.8	3.9	1.4		
	±1.7	±0.8**	±13.3	±7.7**	±81.0	±23.9**	±1.3	±0.3**		
DH	7.0	3.6	74.9	59.7	468.0	114.0	7.9	1.9	857.3	743.0
	±3.4	±3.1**	±12.3	±19.3*	±110.9	±62.3**	±3.6	±0.7**	±371.0	±315.0
:	6.1	6.2	74.6	71.1	346.5	311.4	8.0	8.0	1092.0	987.0
	±3.5	±3.6	±25.7	±19.9	±248.2	±208.6	±5.3	±5.4	±563.0	±436.0

arameters (6 V, 1.6 msec, 6 pulses/sec) were used in all experiments except those in series C, where 4 V, 0.5 msec, 6 pulses/sec were used.
s to Table I for explanation of other symbols.

a unrelated vasodilator, Ach was sub- d for Theo (E in Tables I and II). , the voltage and duration of the is were reduced in order to explore a range of stimulation parameters (C in I and II).

parameters were used to evaluate the e response: Area of the perfusion re response in square centimeters nclosed by the trace and a line drawn he beginning to the end of the re-), maximal fall in perfusion pressure imeters of mercury (mm Hg); initial f perfusion pressure response in mm second; time to 75% recovery of the on pressure response in seconds; and ped muscle tension in grams. Signifi- of difference between means during trol and experimental periods were ed by Student's paired *t* test.

lts. In 11 preliminary experiments (8) not shown in Tables I and II, the basic protocol was followed, but muscle tension was not measured. In these experiments Theo + NE administration completely blocked the responses to all doses of ADO and AMP but was without effect on the responses to all doses of ATP and Ach. It also significantly ($P < 0.005$) reduced the slope of the initial fall in pressure (6.5 to 3.9 mm Hg/sec), the maximal fall in pressure (74 to 51 mm Hg), the duration of the response (575 to 178 sec), and the area of the response (14.9 to 3.2 cm²) during high frequency stimulation, and maximal fall (45 to 23 mm Hg), duration (140 to 66 sec), and area (2.3 to 0.9 cm²) during slow frequency stimulation.

Since it seemed possible that the reduction in exercise dilation resulted simply from decreased exercise, the experiments were repeated while measuring muscle developed

tension and, in some cases, venous P_{O_2}. It is apparent (series B in Tables I and II) that all regularly measured parameters decreased relative to control during infusion of Theo + NE except developed tension. Venous P_{O_2}, measured in five of the eight experiments (not shown in Tables I and II), fell to the same extent during exercise before and during infusion of Theo + NE (the infusion itself had no effect on venous P_{O_2}). The time control series (A in Tables I and II) showd that the changes did not result from spontaneous change in the preparation with time since no parameter during the "experimental" period differed significantly from that in the control period. This was also shown in another way. In three of the experiments in Series B, the muscle was exercised a third time at both the low and high frequencies 20 to 25 min after stopping the infusion of Theo + NE and the responses elicited were not different from those in the control period (the responses were attenuated during Theo + NE infusion in each of the three animals).

The reduced vasodilator responses to exercise were not peculiar to NE administration since they also occurred when ADH was substituted for NE (series D in Tables I and II). Again, developed tension ($n = 7$) and fall in venous P_{O_2} ($n = 6$) in response to exercise were unaffected by the infusion. When the stimulations were repeated a third time after stopping the infusion ($n = 5$), most measures of exercise dilation were not significantly different from those in the control period. The exceptions were response duration and area during slow frequency exercise and response duration during fast frequency exercise which remained significantly reduced (however, in the case of fast exercise, all parameters on the third test were significantly greater than on the second). Neither were the reduced vasodilator responses to exercise peculiar to the administration of a vasodilator in conjunction with NE, since they did not occur when Ach was substituted for Theo (series E in Tables I and II). Finally, the changes also occurred during Theo + NE administration when stimulation voltage and duration were reduced (series C in Tables I and II).

Discussion. In our 1965 study (1), we

found, in preliminary experiments
ırprisingly large attenuation of exer-
ıtion. This led us to conduct a num-
ıdditional experiments in search of
:. Measurements of contractile force
ıous oxygen tension indicated that
nuation of exercise dilation does not
rom decreased muscle activity. Ex-
ıts in the absence of drug administra-
›wed that it does not result from a
eous decline in dilator capacity.
indings indicate that the attenuated
e is unrelated to NE administration;
:ular bed dilated in response to in-
\ch or ATP during Theo + NE ad-
ıtion as well as in its absence and
ted exercise dilation was also seen
Theo + ADH administration. Atten-
was not seen during Ach + NE ad-
ıtion, indicating that Theo is the
l ingredient. Attenuation of the
: response was also seen during
NE administration when the stimu-
›arameters were reduced to levels
lo not cause AMP efflux (10). Fi-
heo did not block ATP vasodilation.
findings suggest that reduction of
: dilation by Theo administration in
ılts from blockade of the vasodilator
›f ADO. Thus, it appears that ADO
ı important role in the exercise dila-
:n at constant flow in the gracilis
of the dog. Additional experiments
: conducted to determine whether
lso the case at natural flow.
r observations also indicate a need
:e study. Both chemical (2, 3) and
y (1, 9) analysis of the venous ef-
rom the muscle mass of the canine
b indicate that the blood contains
ne only during severe or ischemic
:. Furthermore, venous blood from
ine gracilis muscle (perfused at con-
ow) develops enhanced vasodilator
only during moderate to high fre-
exercise (11) and this enhanced va-
r activity can be completely abol-
/ correcting the fall in blood P_{O_2} and
). These findings are not incompati-
ble with the adenosine hypothesis because the adenosine may not always reach the sampling site or bioassay organ in sufficient concentrations for analysis. They do, however, emphasize the need for critical evaluation of the contribution of adenosine to the hyperemia seen under more natural conditions of exercise.

Summary. The canine gracilis muscle was exercised at constant flow before and during administration of theophylline at a rate which completely blocked the vasodilator response produced by injected adenosine. Exercise dilation was significantly attenuated during infusion of theophylline. This suggests a role for adenosine in the exercise dilation seen in the canine gracilis muscle during constant flow perfusion.

The authors acknowledge the able technical assistance of George Gamble and William Stoffs.

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Received June 21, 1976. P.S.E.B.M. 1977, Vol. 154.

Partial Purification and Some Properties (Conjugase) from Chicken

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Most of the folates in the tissues of plants and animals occur in the form of poly-γ-glutamyl peptides having from four to seven glutamic acid residues attached in gamma peptide linkage to the glutamyl moiety of folic acid (1–7). These pteroylpolyglutamates do not support the growth of the commonly used assay organisms (*Lactobacillus casei, Streptococcus faecium,* and *Pediococcus cerevisiae*) employed in the microbiological determination of folates (8). To quantitate the folates present in a biological sample, it is therefore necessary to quantitatively cleave the poly-γ-glutamyl chain to form either pteroylmonoglutamates or diglutamates which the assay organisms can efficiently utilize. This is accomplished by prior enzymatic digestion of the sample employing crude preparations of one of a number of pteroyl-poly-γ-glutamyl hydrolases commonly known by the trivial name of "conjugases." The use of different enzyme preparations obtained from a variety of sources (chicken pancreas, hog kidney, human plasma, etc.) having clearly different properties and often containing significant amounts of contaminating folates, is one of the factors that precludes the direct comparison of results of folate assays obtained by different laboratories. As a consequence, there is now considerable confusion regarding the folate content of natural materials and, most importantly, foodstuffs. For these reasons, it seemed desirable to develop a simple procedure for the extraction and partial purification of a conjugase from a readily available source, resulting in a stable folate-free preparation which would permit a better standardization of the techniques of

[1] The work was supported by Grants No. NO1-HD-4-2862 and No. AM-08644 from the National Institutes of Health, and Grant No. A6249 from the National Research Council of Canada.

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reserved.

l was passed through a DEAE-cel-olumn (2.5 × 20 cm) previously ıted with 200 ml of 0.1 *M* mercap-ɔl. The elution was carried out with n vol of 0.1 *M* mercaptoethanol, or : eluate was free of conjugase activ-conjugase was then further purified ırption and elution from aged alu-'-γ-gel (5% solids content; Sigma al Company). Alumina C-γ-gel and tris-(hydroxymethyl)-aminomethane pH 6.5) in 0.1 *M* mercaptoethanol ıded to the eluate in a ratio of respectively, and the mixture was or 15 min before centrifugation for at 6000 rpm. The supernatant frac-s discarded, and the precipitate was n 20 ml of 0.1 *M* phosphate buffer at for 15 min and centrifuged for 10 000 rpm. The last step was repeated supernates were combined. To pro-table preparation, the supernate was overnight against 20 vol of 2 *M* the nondialyzable remnant was ıoved from the cellophane casing by vith 15 ml of distilled water, lyophi-ıd vacuum-sealed.

ular weight estimation. The molecu-ht of the enzyme was estimated by :x G-75 gel filtration. The column 34 cm) was equilibrated and eluted 1 *M* Tris buffer (pH 6.5) in 0.02% azide and 0.1 *M* mercaptoethanol, ıdardized with ribonuclease A (mol 00), chymotrypsinogen A (mol wt , and ovalbumin (mol wt 45,000). lecular weights of the calibration and the procedures are those given macia Fine Chemicals Calibration cataway, New Jersey).

n determination. Protein concentration was determined by the method of Lowry *et al.* (10) using bovine serum albumin as protein standard.

Paper chromatography. Ascending paper chromatography in 0.2 *M* ammonium acetate buffer (pH 5.8) was used to identify the end product of conjugase action on synthetic pteroylheptaglutamate. Standards of poly-γ-glutamyl derivatives of folic acid (11) were run simultaneously. The folic acid derivatives were detected by uv light absorption.

Results. The summary of purification steps for chicken pancreas conjugase is presented in Table I. The final purification was approximately 50-fold over the activity in the initial mercaptoethanol homogenate, and the yield was 53%. No loss of conjugase activity could be detected after 5 months of storage at −20° of the vacuum-sealed lyophilized preparation. In contrast, the enzyme activity of the DEAE-cellulose eluate decreases gradually with about 50% loss within 1 week at 4°. The alumina C-γ-gel eluate prior to dialysis is even less stable. No detectable folate activity was found in the conjugase preparation when assayed with *L. casei* as the assay organism.

The end product of the enzyme action upon synthetic pteroylheptaglutamate was identified as pteroyldiglutamate by paper chromatography with authentic reference standards.

The elution profile of conjugase activity from Sephadex G-75 shows the presence of two peaks (Fig. 1). The molecular weights of the two components correspond approximately to 50,000 and 25,000, respectively.

The two activity peaks were pooled separately and rechromatographed on a Sephadex G-75 column. The first peak (tubes 30–

TABLE I. SUMMARY OF CONJUGASE PURIFICATION STEPS.[a]

Step	Volume (ml)	Protein (mg)	Total activity (cpm)	Specific activity (cpm/mg protein)	Yield (%)
thanol homogenate	50	2,800	330,000	118	100
thanol extract	83	246	353,320	1,436	107
llulose eluate	250	180	312,500	1,736	95
-γ-gel eluate	30	34.8	175,500	5,043	53

is a typical result with 4 g of Difco chicken pancreas, and all the purification steps are highly ıle.

45) eluted again as a single molecular species in the same position as before. The second peak on the other hand (tubes 46–65) resulted in two peaks of activity essentially identical to the pattern obtained originally (Fig. 1).

The partially purified conjugase also exhibited these two peaks on Sephadex G-75 gel filtration when mercaptoethanol was omitted from both the purification procedure and the elution solvent.

The optimum temperature for conjugase activity from chicken pancreas was between 35 and 50° at pH 8 (shown in Table II). In the pH range 4.0–10.0 at 37°, the enzyme activity increases with pH up to 8.5 and then decreases (Table III).

Discussion. The purification procedure described reproducibly yields a stable preparation of chicken pancreas conjugase which is free of folate and readily obtained in sufficient quantities for use in standardized microbiological assays of folates in natural materials.

The present study confirms the observations of Kazenko and Laskowski (12) and of Jagerstad *et al.* (13) that the product of chicken pancrease conjugase digestion is pteroyldiglutamate. This is also the end product of conjugase purified from chicken intestinal mucosa by Saini and Rosenberg (14).

Our results indicate that the folate conjugase of chicken pancreas consists of two subunits each with a molecular weight of approximately 25,000. Disulfide bridge formation is probably not essential for the formation of the 50,000-molecular weight aggregate, since it is formed in either the presence or absence of mercaptoethanol.

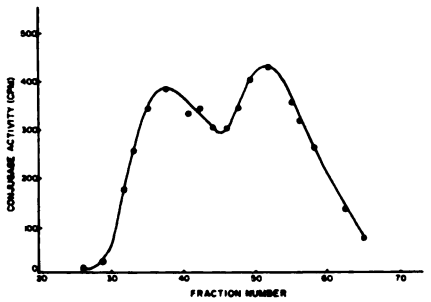

FIG. 1. Elution profile of conjugase activity on Sephadex G-75 (2.5 × 34 cm) with 0.01 Tris buffer (pH 6.5) in 0.02% NaN_3 and 0.1 *M* mercaptoethanol as eluant. Fraction size, 2.0 ml.

TABLE II. CONJUGASE ACTIVITY AS A FUNCTION OF TEMPERATURE IN 0.1 *M* TRIS BUFFER, pH 8.

Temperature (°)	Conjugase activity (cpm)
20	344
35	708
40	722
45	739
50	757
55	696
60	472

TABLE III. CONJUGASE ACTIVITY AS A FUNCTION OF pH AT 37°.

Buffer	pH	Conjugase activity (cpm)
0.1 *M* Na acetate		
	4.0	107
	4.5	136
	5.0	179
	5.5	241
0.1 *M* MES[a]		
	6.0	305
	6.5	389
	7.0	581
0.1 *M* Tris[b]		
	7.5	633
	8.0	706
	8.5	769
	9.0	738
0.1 *M* Borax-NaOH		
	9.3	709
	9.7	18
	10.1	13

[a] (2-[*N*-Morpholine])ethanesulfonic acid.
[b] (Hydroxymethyl)-aminomethane.

Jagerstad *et al.* (13) have also reported that chick pancreas conjugase has a molecular weight of 52,000 by comparison with known standards on gel chromatography, but have observed only a single peak of activity. Although their starting material was the same as that used in the present study, no preliminary purification was used and the analytical procedures differed in several respects. Thus different gel and buffer solutions were used in the two investigations, and different methods were employed to detect enzyme activity. Jagerstad *et al.* (13) used microbiological assay to detect the ability of fractions to convert yeast pteroylpolyglutamate to a form capable of supporting the growth of *L. casei*. It seems

possible that these conditions might not have favored the dissociation of the enzyme or the detection of enzyme activity in subunit fractions.

The enzyme from chicken intestinal mucosa purified by affinity chromatography by Saini and Rosenberg (14) has a molecular weight of 80,000 and no subunits.

The optimum pH for chicken pancreas conjugase is appreciably higher than the optimum pH reported for conjugases from most other tissues (1, 15–17). The latter are primarily lysosomal enzymes with characteristically acidic pH optima. Although the intracellular distribution of chicken pancreas conjugases has not been studied, its high pH optimum argues against its being a lysosomal hydrolase.

Summary. A simple and rapid procedure for the purification of pteroylpolyglutamate hydrolase (conjugase) from chicken pancreas for the purpose of standardization of microbiological assays of folates has been developed. It yields a stable folate-free preparation in quantity. The purification steps included extraction of conjugase from crude lyophilized chicken pancreas in mercaptoethanol followed by DEAE-cellulose chromatography. The enzyme was further purified by absorption to alumina-C-γ-gel from which it was eluted with phosphate buffer, pH 6.5. The enzyme was concentrated by dialysis against 20 vol of 2 *M* sucrose, lyophilized, and vacuum-sealed. The purified conjugase exhibited two peaks on Sephadex G-75 chromatography corresponding to molecular weights of 50,000 and 25,000, respectively. The optimum temperature for the conjugase was between 35 and 50°, and the optimum pH was 8.5.

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Received April 21, 1976. P.S.E.B.M. 1977, Vol. 159.

Serum Lipoprotein Responses to Exogeno of Levels of Dietar

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Diet is considered important in relationship to hyperlipoproteinemia. Although exogenous cholesterol is generally related to diet-induced hyperlipoproteinemia in a number of species, including man, the extent of hyperlipoproteinemia is influenced by other components of the diet as well (1). The role of dietary fat and its interaction with dietary cholesterol has been studied extensively, whereas the effect of dietary proteins, the consumption of which varies widely among different populations, has received less attention. Studies of nonhuman primates and other species have shown conflicting results on the interrelationship of dietary cholesterol and protein in the production of hypercholesterolemia and atherosclerosis (2–8). This study demonstrates the effect of varying the protein content of cholesterol-supplemented diets on the induction of hyperlipoproteinemia in nonhuman primates.

Materials and methods. Animals and diets. The experiment was conducted in two phases. The first phase examined the effect of varying the level of protein intake on the induction of hyperlipoproteinemia in eight adult spider monkeys (*Ateles* Sp.) of mixed sexes. In the second phase, four more adult spider monkeys were added to the original group to study the effect of methionine in very low protein diets on serum lipid levels.

Prior to being fed the experimental diets, the animals received a regular diet of Purina Monkey Chow 25, which is approximately 55% carbohydrate, 25% protein, and 5% fat (w/w). The experimental diets were arbitrarily chosen to contain high [25% (w/w)], low [8% (w/w)], or very low [4% (w/w)]

[1] Supported by funds from the National Heart and Lung Institute of the USPHS, Specialized Center of Research-Arteriosclerosis (SCOR-A), No. HL15103 and No. HL02942.

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TABLE I. DIETARY COMPOSITION.

ngredients (g/100 g)	High protein (25%)		Low protein (8%)		Very low protein (4%)[a]	
	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI
how 25[b]	41.0	38.0	–	–	–	–
how 15[b]	–	–	53	52.5	27.0	27.0
	15.0	15.0	–	–	–	–
	–	5.0	–	5.0	–	5.0
	–	–	–	–	1.0	1.0
	–	–	–	–	30.0	20.0
salt	–	–	–	–	1.0	1.0
	–	–	–	–	1.0	1.0
	12.0	12.0	15	10.0	11.0	13.5
rol[c]	–	0.5	–	0.5	–	0.5
	32.0	29.5	32.0	32.0	29.0	31.0
ergy (kcal/g)	2.48	2.72	2.36	2.71	2.50	2.54

hionine-supplemented diets were prepared by adding 0.4% of the amino acid to diets V and VI.
na chow contains 25 and 15% (by weight) protein.
talline cholesterol was dissolved in melted butter and mixed with other ingredients.

ı studying the serum lipoproteins of ıt nonhuman primate species (13). fference between total cholesterol + pre-β-lipoprotein cholesterol was red as α-lipoprotein cholesterol.

itometric ratios of β- and pre-β-lipo-. At the end of each dietary period -first day), electrophoresis of serum teins was done on an agar–agarose ording to the method of Noble (14) me modification (15). The lipopro-nds were scanned in a Quick Scan meter (Helena Laboratories, Beau-Tex.), and the densitometric ratios of pre-β-lipoproteins were corrected as ed previously (16).

ıation of β-, pre-β-, and α-lipopro-. ssuming that cholesterol content per · or pre-β-lipoprotein molecule in ys is similar to that of humans, we ted the serum content of these two of lipoproteins based on the densito-ratios of β- to pre-β-lipoprotein, and re-β-lipoprotein cholesterol content). The method was validated by com-ts results with those obtained by ana-ıltracentrifuge, and the results were ement (11). Serum levels of α-lipo-s were obtained by multiplying the rotein cholesterol value by a factor 16).

sterol response index. The serum rol response to dietary cholesterol asured in terms of cholesterol re-index (mg/100 ml) as described by Bowyer *et al.* (17) with some modifications. This index was obtained by dividing the area under the curve of serum cholesterol concentration, above the basal level, by the duration (days) of feeding of cholesterol-supplemented diet. The basal value for each dietary protein level represented the mean of three determinations obtained at days 14, 17, and 21 during the corresponding basal diet period. Similar indices were calculated for β + pre-β-lipoprotein cholesterol and α-lipoprotein cholesterol.

Serum albumins. Serum albumin assay was done according to the Auto-Ref procedure manual (DADE Division of American Hospital Supply Corp, Miami, Fla.), which is a modification of the standard ether–sulfate method (18).

Histology. The liver biopsies were done using a Vim-Silverman needle. The tissues for light microscopy were fixed in 10% Formalin and processed in paraffin; sections were cut at different levels and stained with hematoxylin and eosin.

Results. The monkeys continued to maintain body weight with no observable ill effects for the duration of the study. When the animals were fed 25, 8, and 4% protein diets, their respective mean ± SD body weights were 6.18 ± 0.78, 6.05 ± 0.99, and 6.28 ± 0.75 kg.

Although the basal serum cholesterol and β + pre-β-lipoprotein cholesterol concentrations in these monkeys tended to decrease slightly with reduced intake of die-

tary protein, the differences were not statistically significant (Table II). Alpha-lipoprotein cholesterol decreased slightly but significantly ($P < 0.05$) when the protein intake was reduced from 25 to 8%. Interestingly, however, further reduction in dietary protein intake to a 4% level caused a highly significant ($P < 0.001$) increase in α-lipoprotein cholesterol. The serum concentrations of the above variables for each level of dietary protein did not change significantly by changing the dietary sequence from a high protein to a low protein diet or vice versa over these short observation periods.

As expected, the animals responded to exogenous cholesterol, but responses varied at the different levels of dietary protein (Fig. 1). For instance, the 25% protein diet gave a relatively higher serum total cholesterol response (28.1 ± 9.2 mg%) than the 8% protein diet (18.7 ± 10.7 mg%). On the other hand, further reduction in dietary protein to the 4% level markedly increased the serum total cholesterol response (51.5 ± 17.6 mg%). Serum cholesterol response to exogenous cholesterol was reflected predominantly in β + pre-β-lipoprotein cholesterol fraction when the animals were on 25% diet (25.4 ± 8.7 mg%) and 8% diet (14.6 ± 7.8 mg%), and the values differed significantly ($P < 0.05$) during the two dietary periods. Both β + pre-β-lipoprotein cholesterol (29.7 ± 12.8 mg%) and α-lipoprotein cholesterol (21.9 ± 6.1 mg%) responses increased markedly upon 4% protein intake. An analysis of variance of the responses of serum total cholesterol ($P < 0.001$), β + pre-β-lipoprotein cholesterol ($P < 0.01$), and α-lipoprotein cholesterol ($P < 0.001$) to a high cholesterol (0.5%) diet showed a significant difference due to the level of dietary protein.

TABLE II. SERUM LIPOPROTEIN CHOLESTEROL LEVELS (MEAN ± SD) IN SPIDER MONKEYS FED DIETS WITH VARYING PROTEIN CONTENT.[a]

Cholesterol fraction (mg/100 ml)	Dietary protein level (g/100 g)		
	25	8	4
Serum total cholesterol[b]	177.6 ± 34.5	167.2 ± 23.4	164.9 ± 20.8
β + pre-β-lipoprotein cholesterol[c]	139.5 ± 37.4	133.2 ± 29.9	110.4 ± 16.4
α-lipoprotein cholesterol[d]	38.1 ± 7.5[e]	34.0 ± 8.5[f]	54.5 ± 6.9[g]

[a] Represents three determinations during each dietary period (diets I, III, and V) for each animal ($N = 8$). $e > f$ ($P < 0.05$); $e < g$ ($P < 0.001$); $f < g$ ($P < 0.001$).

[b] Measured by Auto Analyzer II (9).

[c] Measured by heparin-Ca^{2+} precipitation method (10, 11).

[d] Difference between total cholesterol and β + pre-β-lipoprotein cholesterol.

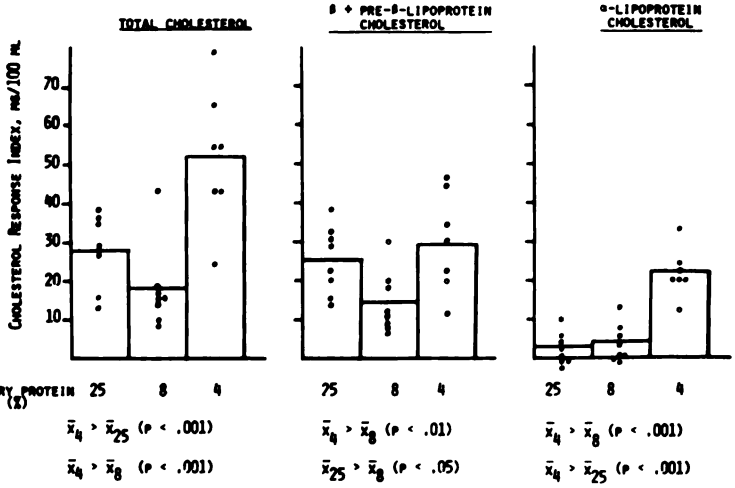

FIG. 1. Serum lipoprotein cholesterol response in spider monkeys fed high cholesterol diets containing different levels of protein.

III. Serum Cholesterol and Lipoprotein Levels (Mean ± SD) in Spider Monkeys Fed High Cholesterol Diets[a] Containing Different Levels of Protein.

0 ml)[b]	Dietary protein level (g/100 g) 25	8	4	Analysis of variance
olesterol	340.4 ± 69.1	290.1 ± 61.7	534.9 ± 140.3	$P < 0.001$
rotein	558.4 ± 155.5	461.6 ± 184.9	754.7 ± 251.0	$P < 0.05$
oprotein	25.1 ± 15.9	33.9 ± 19.5	27.4 ± 11.3	$P > 0.10$
rotein	430.4 ± 223.4	402.6 ± 203.4	1035.0 ± 363.0	$P < 0.01$

:-tenths gram of cholesterol/100 g diet (diets II, IV, and VI).
ıse refer to the text for the methods of measurements of these variables.

le III shows the means of serum total terol and lipoprotein concentrations terminus of 3-week feeding of high terol diets and the different levels of ı. Unlike other serum lipid variables, lipoprotein showed no significant : due to exogenous cholesterol and to /el of dietary protein intake. Therehe observed changes in serum cholest different levels of dietary protein are ed in β- and α-lipoproteins.

ıough methionine-supplemented diets ed the increased serum cholesterol re- to exogenous cholesterol by 25%, its was not uniform. One-third of the :ys continued to show an increased se to dietary cholesterol in spite of ınine supplementation. Changes in β + pre-β-lipoprotein cholesterol -lipoprotein cholesterol in these aniıowed trends consistent with the cho- l responses.

ım albumin levels were measured as a mical parameter for protein-calorie trition. The albumin levels (g/100 ml) ıkeys fed 25% protein, 8% protein, % protein with added methionine .48 ± 0.36, 5.21 ± 0.68, and 5.31 ± espectively, and the statistical analyıwed no significant differences. Furıre, liver biopsy material examined a light microscope showed no fatty ıaracteristic of protein-calorie malnu-

ussion. Although the level of dietary ı per se had little appreciable primary on serum lipids, different levels of ı intake clearly had a varied interactect on the serum lipid responses to ıous cholesterol in spider monkeys. and McGill (3) found that 25% proets tended to produce slightly higher serum cholesterol levels in baboons than did 10% protein diets, and they suggested a second-order interaction among dietary cholesterol and level of protein in the diet. In contrast, Middleton *et al.* (2) reported that the level of dietary protein had no interacting effects on serum cholesterol levels in squirrel monkeys fed high cholesterol diets.

As in previous studies, the serum cholesterol response to exogenous cholesterol was reflected in β- as well as α-lipoproteins during the transient phase from basal diets to ones with added cholesterol (12-19). The marked increase in α-lipoprotein cholesterol during the very low protein diet with and without cholesterol added is of particular interest. Further lipoprotein characterization studies are needed to clarify this response.

Underlying reasons for the varied effects of dietary protein intake on serum cholesterol and lipoproteins are not established. Synthesis of liver-produced plasma proteins is regulated by dietary protein and amino acid supply (20). Certain amino acids from dietary proteins are utilized for "extra protein functions" as well as for energy metabolism and protein synthesis (21-23). Methionine is required as a substrate in transmethylation reactions and production of choline for the phospholipids. Since a deficiency of sulfur amino acids impairs the efficiency of regulation of cholesterol in the blood (24), any reduction in sulfur amino acids in the diet could greatly increase the serum cholesterol response to exogenous cholesterol. Although the effect of adding methionine to the very low protein diet was obvious, it was not consistent on serum lipids in all the animals. The absence of fatty livers and no change in serum albumin levels suggest that a 4% protein level may not be low enough

to produce protein–calorie malnutrition detectable by these parameters in this period of time. Enwonwu *et al.* (25) observed that nonhuman primates develop characteristic clinical and biochemical features of protein–calorie malnutrition only at 2% protein level (w/w).

Isocaloric substitution of the bulk of the proteins with carbohydrate (dextrin) in the very low protein diets is another factor to consider in explaining the pronounced hyperlipoproteinemia (26–28). It should be noted that the very low protein diet also was lowest in nondigestible fiber. The high and the very low protein diets used in this study might represent an exaggeration of the protein level in the usual human diet in the United States, which ranges from 15 to 20% of total calories, whereas the 8% protein diet representing 12% of the calories, may be close to the optimal level. Low protein levels generally contain low levels of fat and cholesterol; populations consuming such diets show relatively low levels of serum cholesterol and a lower incidence of coronary artery disease.

Summary. The interacting effects of high (25%), low (8%), and very low (4%) dietary protein on the serum lipoprotein concentrations have been studied in spider monkeys (*Ateles* Sp.). Although the level of dietary protein per se had no appreciable main effect on the basal levels of serum total cholesterol, very low protein diet produced significant elevation in α-lipoprotein cholesterol. The serum lipoprotein responses to exogenous cholesterol varied at different levels of protein intake, the degree of response being highest at 4% protein level and lowest at 8% protein level. The changes were observed mainly in β- and α-lipoproteins.

The authors appreciate the histologic examinations of liver biopsy done by Dr. William Newman, Jr., of the Department of Pathology, and the technical assistance of Mr. Charles C. Smith.

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Received July 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 107-111 (1977)

Developmental Changes of Immunoglobulins in Rats Treated Neonatally with Hydrocortisone[1] (39614)

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Immunodeficiency states can develop through a variety of mechanisms. Genetically determined immunodeficient mice have been described and investigated extensively. The Ames dwarf mouse exhibits lymphopenia, involution of thymic and peripheral lymph tissue, and a wasting syndrome. The serological response to sheep erythrocytes (RBC) is delayed and decreased in the dwarf, as is the graft vs host response. However, marked deficiencies in resting serum immunoglobulins are absent. These findings have been interpreted as an immunodeficiency state restricted to the thymic system (1). Nude mice exhibit a different immunodeficiency syndrome, though they also have wasting syndrome and decreased serological response to challenge with sheep RBC. IgM levels are normal in comparison to controls, while IgG and IgA are low (2).

Deficiencies in the immune system have been produced by administering adrenal cortical hormones. The subsequent thymic involution first was described by Dougherty and White (3), and since has been confirmed by others. Recently, Greengard and Machovich (4) have found decreased levels of thymic thymidine kinase and increased levels of L-glutamyl-transferase and glutamine synthetase. Levels of six other enzymes were unaltered. These studies may provide a biochemical basis for the effects of glucocorticoids on thymic size. Halpern *et al.* (5) report that daily injections of cortisone, beginning at Day 5, impair survival and are associated with decreased antibody response to typhoid bacilli and with lower gamma-globulin levels by 21 days of age. In rats, a single injection of 1 mg of cortisol acetate within 24 hr of birth is correlated with decreased passive hemagglutination titers of *Salmonella typhosa* and decreased serological response to sheep RBC in 60 to 70-day-old rats (6).

The immunological change produced by neonatal injection of glucocortocoids suggests that a model of immunodeficiency which may resemble one of the clinically described diseases in man could be produced by this method. Thus, further study of the immunological consequences of neonatal glucocorticoid injection seems appropriate. The present study attempts to characterize them further.

Methods and materials. Pregnant Sprague-Dawley rats from our own colony were used. Within 24 hr after birth, pups were injected sc with 20 μl of cortisol acetate (Upjohn, Cortef acetate, 50 mg/ml). We refer to these rats as corticoid runts. Controls were injected sc with an equal volume of 0.9% NaCl. Litters were culled to a maximum of 10 animals. After treatment, rats were returned to their mothers until sacrifice or weaning at 24 days of age. Animals were maintained at 25°; Purina rat chow and water were provided *ad libitum*.

Rats were decapitated at 3, 6, 11, 15, 20, 30, and 60–75 days of age. Blood was collected and centrifuged, and the serum was removed. Serum from very young rats was pooled so that each sample contained approximately 0.5 ml. Samples were frozen at 0°.

Globulins were isolated from pooled rat serum (7). The isolated material contained 6.58 mg/1 ml as determined by the method of Lowry (8). Rabbit anti-rat globulin was obtained from Gibco (Grand Island, New York). Rat IgG and rabbit anti-rat IgG were purchased from the Pentex Division of Miles Laboratories (Kankakee, Ill.). The

[1] Supported by USPHS Grants No. HD8575 and No. AM17594.

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vendor states that the rabbit anti-rat IgG is immunospecific, and we have verified this by immunoelectrophoresis against rat serum.

Serum levels of globulin and IgG were measured by agar-gel diffusion against anti-rat globulin and anti-IgG on immunodiffusion plates (Pattern C, Hyland Laboratories, Costa Mesa, Calif.) with serial dilutions of standards run daily. One-third of the samples were run in duplicate. The endpoint of the dilutions for IgG and globulin was designated as equivalent to one unit and serum concentrations were calculated accordingly. Statistical comparisons were made by Student's *t* test.

To investigate immunoglobulin absorption from the intestinal tract, five samples of 100 μg of IgG were dissolved in 100 μl of phosphate buffer, pH 7.4, and iodinated with 60 μCi of ^{125}I (Amersham Searle), as described by Yalow and Berson (9). Each sample was purified on a Sephadex G-50 column, 1.2 by 5 cm, connected to an LKB fraction collector recorder. Protein was monitored by ultraviolet absorption. Fractions containing protein were pooled. The free ^{125}I in the purified preparation was estimated from the radioactivity remaining in solution after deproteinization as compared to the total radioactivity of the sample. To that end, 100 μl of each sample was directly dissolved in 1 ml of soluene in a counting vial. A second 100 μl of each sample was treated with 150 μl of 10% trichloroacetic acid, centrifuged, and 100 μl of the protein-free supernatant was similarly added to a vial, followed by 1 ml of soluene. Ten milliliters of Bray's solution was added to each vial and radioactivity was determined in a liquid scintillation counter. Since free iodine in the five samples averaged only 13.3% ± 4.1 (standard error), the samples were not further purified, but were pooled, lyophilized, and dissolved in 0.9% NaCl.

At 9 AM the following day, the mothers were removed from one litter of controls and one litter of corticoid runts. All pups were 13 days old. One hour later, 0.2 ml of [^{125}I]IgG was intubated into each pup. Two control rats were intubated with saline only. Three hours later, animals were decapitated, the blood collected, and the serum removed. One-hundred or two-hundred microliters of serum was solubilized with 10 vol of soluene, 10 ml of a soluene-based scintillation cocktail was added, and the samples, together with a vial containing 100 μl of the [^{125}I]IgG, were counted. Quench corrections were made by recounting the vials after addition of 0.1 ml of a mixture of 150 μl of [^{125}I]IgG and 1.6 ml of soluene.

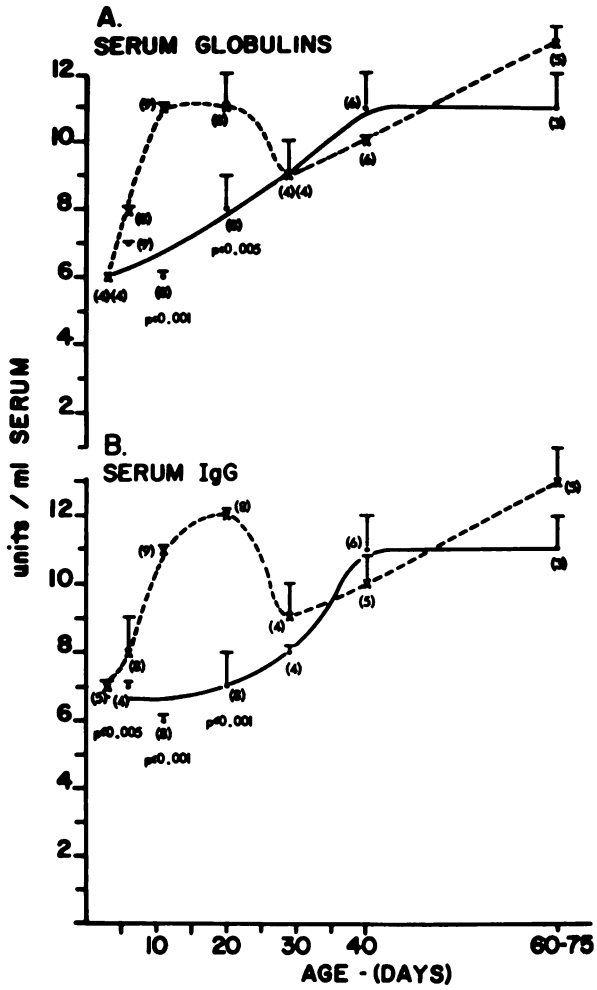
To determine the fraction of ^{125}I bound to the rat serum proteins, the serum from all rats intubated with radioactive IgG was pooled and 0.3 ml was taken to estimate total radioactivity. Unlabeled albumin was added to a second 0.3-ml aliquot to act as carrier, protein was precipitated with 1 ml of 10% trichloroacetic acid, the sample was mixed and centrifuged, and 0.5 ml of the supernatant was used to determine unbound ^{125}I.

To investigate antibody synthesis, rats were sensitized by intraperitoneal and intradermal injections of 10% sheep red blood cells (SRBC) on Days 14 and 18. The animals were exsanguinated 1 week after the last injections and their spleens were removed. Hemolytic direct plaques were measured as described by Levy and Harris (10). Hemagglutination titers were measured in microtiter plates using mercaptoethanol pretreatment to identify the relative amounts of IgG and IgM by the method of Braley-Mullen (11).

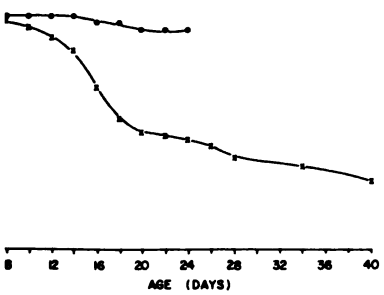
Survival rates were evaluated by counting the number of rats remaining in five litters of corticoid runts and three liters of controls. Litters were checked every 3 to 4 days. The percentage of the total surviving at a given age is shown in Fig. 2.

Results. Developmental changes in total globulins and IgG in corticoid runts and controls are shown in Fig. 1. Control levels of globulins rise steadily in the suckling rat and reach a peak at 11 days. Levels remain high until after weaning. Although levels are lower at 30 days than at 20 days, they are not as low as in newborn rats. Between 30 and 75 days, levels rise gradually. Serum globulins in newborn corticoid runts rise very slowly during development, so that at 11 days they are significantly lower than levels in controls and remain down until 30 days. Thereafter no significant difference is found between the two groups.

Serum IgG levels show a similar trend. In



Developmental changes in concentration of (A) serum globulins, and (B) IgG levels in controls and in corticoid runts (●——●). Mean ± SE is shown at each age. Sample size is in parentheses.



Comparison of survival rates in controls and in corticoid runts (×---×).

they rise rapidly until the animals lays old. After weaning, they fall usly and then gradually rise. By serum IgG levels in runts remain low throughout the suckling period. At 6 days, they are significantly lower than in controls and remain so until after weaning, when they begin to rise slowly. Levels peak at 40 days and do not rise further.

Absorption of IgG from the gastrointestinal tract is expressed in counts per minute per 100 μl of serum per 0.1 ml of [^{125}I]IgG. At 13 days of age, the mean ± standard error in four untreated rats is 527 ± 138; in three corticoid runts, it is 593 ± 29 ($P > 0.7$). In rats intubated with saline levels were 57 and 67. Free iodine levels in the intubation mixture of [^{125}I]IgG were 13.3%. The free iodine in the pooled sera of intubated rats was 22%.

The immunological response to t-depend-

TABLE I. EFFECT OF NEONATAL HYDROCORTISONE T AT 25 DAYS

Group	IgG +
Controls	1.2861 ± 0.(
Corticoid runts	0.7525 ± 0.1

[a] Mean ± SE. Sample size is in parentheses.
[b] Significantly less than control titer ($P < 0.01$).

TABLE II. EEFFECT OF NEONATAL HYDROCORTISONE AGI

Group	Pla
Controls	1062
Corticoid runts	108

[a] Mean ± SE. Sample size in parentheses.
[b] Significantly less than control titer ($P < 0.005$).

ent SRBC (Tables I and II) demonstrates a severe depression in the corticoid runts' ability to produce antibodies. The hemagglutination assay (Table I) shows that the control rats had a titer of 1:16 (IgG + IgM) and 1:6 of IgG alone while the steroid-treated animals had a titer of 1:6 (IgG + IgM) and 1:2 of IgG alone. This depression in antibody formation also is reflected in the plaques either on the basis of number of lymphocytes studied or on the basis of spleen weight (Table II). Essentially, in either case, the corticoid runts only had 10% of the plaques seen in the controls. The runts were somewhat smaller, but proportionally they had a greater spleen to body weight ratio of 6.27 ± 0.35 (mean ± SE) than controls (4.62 ± 0.44). Units are in milligrams per gram of body weight.

Survival rates of both controls and corticoid runts are seen in Fig. 2. By 24 days of age, 94% of controls were alive, but less than 50% of the runts had survived; in fact, 94% survived only until Day 10. Between Days 12 and 18, survival of corticoid runts fell rapidly from 90 to 55%. Thereafter the drop was less dramatic, although by 40 days only 30% of the runts were alive. Growth is severely retarded in the runts, as evidence by persistently lower body weight as described previously (12). Generally, they appear less active than controls and more prone to diarrhea and eye infections.

Discussion. At least part of the decreased survival of runts may be due to their limited

adult. Our findings demonstrate that this deficit is present prior to weaning. Hence, neonatal corticoid treatment produces immediate effects on immunological capacity which persist into adulthood.

Halpern *et al.* (5) also report decreased survival and failure of antibody production in young rats treated daily with cortisone. However, the simplicity of the single injection we used in the present study in producing chronic defects is very appealing.

Summary. Serum globulin and IgG levels were measured by radial immunodiffusion from 3 to 75 days after birth in rats neonatally treated with 1 mg of cortisol acetate. In controls, globulins rose steadily during suckling, peaked at 11 days, and remained high until weaning, when they dropped considerably. Thereafter, they rose steadily until 75 days. In treated animals, levels increased slowly during development and were significantly lower than controls until 30 days. Serum IgG followed a similar pattern. The most severe immunological deficit in runts began on Day 12. The most marked mortality in treated rats also began at this age and in part may be due to the severe immunological impairment. No difference was detected in absorption of IgG from the gastrointestinal tract of the two groups. However, dramatic deficits were demonstrated in the ability of treated rats to synthesize antibody when sensitized with a t-dependent antigen. Thus, the difference in immunological competence is due to endogenous deficits produced by a single injection of cortisol acetate at birth.

We gratefully acknowledge the technical assistance of Willie Mills, Peggy Cohen, and David Chia.

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Received December 2, 1975. P.S.E.B.M. 1977, Vol. 154.

Increased Pulmonary Vascular Pressor I (396

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Several low altitude laboratories have reported much smaller increases in pulmonary vascular resistance induced by acute hypoxia in dogs (1–3) than those which we have observed at 1600-m altitude (4). We wished to determine if this poor hypoxic response in lowland dogs was due to differences in technique, or due to a real physiologic difference. The mechanism by which alveolar hypoxia causes pulmonary arterial vasoconstriction remains unknown. If lowland and highland dogs differ in their response to hypoxia, further studies of these two groups might shed light on this mechanism. Therefore, we studied the pulmonary pressor response to hypoxia in lowland and highland dogs under identical climatic and laboratory conditions.

Methods and materials. Fourteen mongrel dogs of either sex (mean weight 19 ± 1 kg) were flown to Denver, Colorado (1600 m), from sea level laboratories in New Orleans, Louisiana (n = 8), and Madison, Wisconsin (n = 6) (lowland dogs). Ten dogs were studied the day after arrival, while the remaining four dogs were studied 4 (n = 2) or 7 (n = 2) days later. At autopsy, three of the dogs from New Orleans were found to have numerous heart worms (*Dirofilaria immitus*) in the right ventricle and main pulmonary artery. Consequently, these dogs were excluded from the comparison made with 11 "highland" mongrel dogs (mean weight 21 ± 1 kg) obtained in Denver. We assumed that the dogs were residents of the altitude from which they were obtained.

All of the dogs were studied using the same procedures. They were anesthetized with intravenous sodium pentobarbital (30 mg/kg) and intubated with a cuffed endotra-

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blood gas tensions and pH were measured with a Radiometer blood gas analyzer (Model 27) at 38° and corrected to the animals' deep body temperature.

The hypoxic challenge was maintained for 20 min. Vascular pressures were measured every min, cardiac output at 5, 10, 15, and 20 min, and arterial blood gases at 10 and 20 min of hypoxia. Twenty minutes after the end of the hypoxic exposure, the pulmonary arterial pressor response to an intravenous bolus of prostaglandin $F_{2\alpha}$ was recorded. This response was observed in order to determine if any difference between the groups was purely associated with hypoxic vasoconstriction, or whether it also involved other constrictor stimuli. A dose of 5 μg/kg was chosen because we have found, in other experiments, that this is the lowest dose which will provoke a maximum increase in pulmonary arterial pressure.

Seven of the lowland dogs were allowed to recover from anesthesia and were restudied, in exactly the same manner, between 3 and 4 weeks after the initial experiment. These experiments were conducted to identify any changes in response induced by residence at 1600 m. In addition to the dogs from Denver and sea level, three dogs from Breckenridge, Colorado (approximate altitude 2700 m), were also studied.

The data are given in the text and tables as the mean and 1 SE. The differences between lowland and highland dogs, in terms of their control values and responses to hypoxia, were analyzed with the two population t test. Changes occurring in the lowland dogs that were restudied were examined using the paired Student's t test. Significant differences were considered to exist when $P < 0.05$.

Results. There were no significant differences between the lowland and highland dogs in the hemodynamic variables measured during normoxic conditions. However, the increases in pulmonary arterial pressure and pulmonary vascular resistance induced by hypoxia were greater in the highland dogs (Table I, Fig. 1). The percentage increase in pulmonary vascular resistance was also greater in the highland dogs (+135% ± 17) than in the lowland dogs (+65% ± 7). The severity of the hypoxic challenges was the same as evidenced by the similar blood gas tensions during hypoxia (Table II). Cardiac output and systemic arterial pressure increased during hypoxia in both groups. In the lowland dogs there was a significant fall in systemic arterial resist-

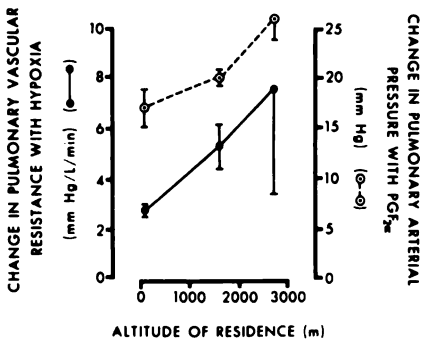

FIG. 1. Change in pulmonary vascular resistance during hypoxia (closed circles and unbroken line) and in pulmonary arterial pressure with prostaglandin $F_{2\alpha}$ (a bolus of 5 μg/kg, open circles and broken line) in dogs native to sea level (n = 11), Denver (1600 m) altitude (n = 11), and 2700 m (n = 3).

TABLE I. NORMOXIC HEMODYNAMICS IN LOWLAND AND HIGHLAND DOGS AND CHANGES WITH ACUTE HYPOXIA.[a]

	Normoxic control		Change with hypoxia	
	Low	High	Low	High
Pulmonary arterial pressure (mm Hg)	16 ± 1	17 ± 1	+12 ± 1	+17 ± 1*
Pulmonary vascular resistance (mm Hg/liter/min)	4.5 ± 0.3	4.0 ± 0.4	+2.8 ± 0.2	+5.3 ± 0.9*
Cardiac output (liter/min)	2.7 ± 0.2	3.3 ± 0.3	+0.7 ± 0.1	+0.2 ± 0.2
Systemic arterial pressure (mm Hg)	134 ± 3	136 ± 6	+13 ± 2	+9 ± 3
Total systemic resistance (mm Hg/liter/min)	51 ± 3	44 ± 4	−6 ± 2	0 ± 2*

[a] Low, lowland dogs (n = 11); high, highland dogs (n = 11). * Difference in hypoxic response between lowland and highland dogs is significant at $P < 0.05$ level.

TABLE II. ARTERIAL BLOOD GAS TENSIONS DURING NORMOXIA AND HYPOXIA IN LOWLAND AND HIGHLAND DOGS.

	Normoxia		Hypoxia	
	Low	High	Low	High
Systemic arterial pH	7.32 ± 0.01	7.32 ± 0.01	7.36 ± 0.01	7.36 ± 0.01
Systemic arterial oxygen tension (mm Hg)	97 ± 2	99 ± 2	38 ± 1	39 ± 1
Systemic arterial carbon dioxide tension (mm Hg)	41 ± 1	41 ± 1	39 ± 1	39 ± 1

TABLE III. NORMIC HEMODYNAMICS IN LOWLAND DOGS AND CHANGES WITH ACUTE HYPOXIA AFTER RESIDENCE AT 1,600 m.

	Normoxic control		Change during hypoxia	
	First study[a]	Second study[b]	First study	Second study
Pulmonary arterial pressure (mm Hg)	16 ± 1	16 ± 1	+12 ± 1	+12 ± 1
Pulmonary vascular resistance (mm Hg/liter/min)	4.7 ± 0.4	4.2 ± 0.8	+2.7 ± 0.3	+2.9 ± 0.6
Cardiac output (liter/min)	2.7 ± 0.2	3.2 ± 0.4	+0.8 ± 0.2	+0.5 ± 0.1

[a] Study on arrival at 1600 m from sea level; $n = 7$.
[b] Study 3–4 weeks later; $n = 7$.

ance in comparison to the highland dogs.

The increase in pulmonary arterial pressure induced by prostaglandin $F_{2\alpha}$ was not significantly different in the two groups. The increase was +17 ± 2 mm Hg in the lowland dogs, and +20 ± 1 mm Hg in the highland dogs (Fig. 1). The pulmonary arterial pressures prior to prostaglandin $F_{2\alpha}$ administration were 15 ± 1 and 16 ± 1 mm Hg, respectively.

Seven lowland dogs were restudied 3 to 4 weeks after the initial experiment. Their pulmonary arterial pressures and resistances during normoxia, and the changes with hypoxia were not different from the values obtained during the first study (Table III). Similarly, the change in pulmonary arterial pressure induced by prostaglandin $F_{2\alpha}$ was unaltered (15 ± 1 to 33 ± 2 mm Hg during the first study, and 15 ± 2 to 33 ± 3 mm Hg during the second study).

The three dogs from 2700 m also exhibited greater pulmonary vascular reactivity (Fig. 1). During hypoxia the mean increase in pulmonary vascular resistance was +7.6 ± 4.2 mm Hg/liter/min (+139% ± 39). While this increase is somewhat greater than the increase observed in the Denver dogs, the percentage change in resistance was not different. The increase in pulmonary arterial pressure stimulated by prostaglandin $F_{2\alpha}$ was +26 ± 2 mm Hg (from 15 ± 1 to 41 ± 3 mm Hg), which also tended to be greater than the response observed in the Denver dogs (+20 ± 1 mm Hg).

Heart worm infestation ($n = 3$) increased both the normoxic pulmonary arterial pressure (21 ± 3 mm Hg) and pulmonary vascular resistance (6.4 ± 1.4 mm Hg/liter/min). The absolute increases during hypoxia (+12 ± 1 mm Hg, and +2.5 ± 1.3 mm Hg/liter/min) were similar to those for the remainder of the lowland group; however, the responses expressed as percentage change were smaller.

Discussion. This study demonstrates that both in terms of pulmonary arterial pressure and calculated resistance the highland dogs had greater responses to hypoxia than lowland dogs, suggesting that chronic low-grade hypoxia may alter hypoxic reactivity. The greater response of the highland dogs to hypoxia is in contrast to our experience in rats (5) and cattle (6). Lungs isolated from rats which had been exposed at 4270 m for 5 weeks showed less pulmonary hypertension in response to acute alveolar hypoxia than those from control rats maintained at 1600 m. Hypoxic pulmonary hypertension, with associated right heart failure (brisket disease) is less common in cattle native to altitudes above 2133 m than in lowland cattle

taken to high altitude. We have shown that the characteristic to susceptibility or resistance to hypoxic vasoconstriction is inherited (7). This is true of the acute or chronic response to hypoxia (8). It is thought that natural selection through a number of generations may have rendered the highland cattle more resistant to the pulmonary vascular effects of hypoxia (9). Dogs do not develop chronic pulmonary hypertension at high altitude (10), and increased responsiveness of the pulmonary vascular bed would not therefore be a detrimental factor in natural selection. Whatever the change may be, increased vascular reactivity appears to be either acquired over a long period of time (certainly in excess of 4 weeks), or else inherited.

The pressor response to prostaglandin $F_{2\alpha}$ tended to be greater in the dogs from 1600 m than in those from sea level, and was even greater in dogs from 2700 m than in those from the two lower altitudes. This suggests an increasing responsiveness to prostaglandin $F_{2\alpha}$ with increasing altitude of residence. The observation is consistent with the greater responsiveness to prostaglandin $F_{2\alpha}$ in lungs from high vs low altitude rats (5).

It is evident from this study that the altitude from which dogs are obtained is yet another variable which should be considered when hemodynamic results from various laboratories are compared. Perhaps the different pulmonary vascular pressor responses seen in the highland and lowland dogs will provide a stimulus and a model for the study of the mechanism of hypoxic pulmonary vasoconstriction.

Summary. Greater pulmonary vascular resistance increases in response to acute hypoxia have been observed in the anesthetized dog at 1600-m altitude than in dogs studied at sea level. Consequently, the hemodynamic response to hypoxia was studied in 11 "lowland" dogs flown to Denver and compared to the response of 11 dogs obtained locally. The local "highland" dogs had mean increases in pulmonary arterial pressure and pulmonary vascular resistance during hypoxia of +17 ± 1 mm Hg and +5.3 ± 0.9 mm Hg/liter/min, respectively, in contrast to the increases of +12 ± 1 mm Hg and +2.8 ± 0.2 mm Hg/liter/min observed in the lowland dogs. The increase in pulmonary arterial pressure induced by prostaglandin $F_{2\alpha}$ was not significantly different in the two groups. The pulmonary pressor response to hypoxia in seven lowland dogs restudied after 3- to 4-weeks residence at 1600 m was not altered. The difference in responsiveness of the pulmonary vascular bed to hypoxia in the two groups suggests that chronic low-grade hypoxia may alter hypoxic reactivity, and that the resident altitude should be considered when comparing data from various laboratories.

We are very grateful to Dr. A. L. Hyman and Dr. P. J. Kadowitz (Louisiana) and Dr. J. A. Will (Wisconsin) who supplied the sea level dogs for the study. Excellent technical assitance was provided by Mary Munroe, Rosann Glas, Steve Hofmeister, Bruce Hookway, Don Jackson, Bea Kaplan, and Eva Toyos. The manuscript was prepared by Karen Leahy. Prostaglandin $F_{2\alpha}$ was kindly supplied by Dr. John Pike, Upjohn Co., Kalamazoo, Mich. The study was supported by NIH Grants No. HL 14985 and No. HL 05973.

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Received June 23, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 116-120 (1977)

Effects of Prolonged Fenfluramine Administration in Obese and Nonobese Mice (39616)

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The amphetamine derivative, fenfluramine (*N*-ethyl-α-methyl-3-trifluoromethyl-β-phenylethylamine), produces anorexia in man and other animals without the central nervous system stimulation produced by the parent amphetamine (1). While the majority of studies in humans has involved obese patients, most of the animal experiments have been performed in normal weight animals, although a few studies were done in rodents made obese by electrical (2) or chemical (3) lesioning of the hypothalamus. Although these investigations have been principally concerned with the anorectic action of fenfluramine, a number of metabolic effects has also been reported, including increased glucose uptake by muscle (4), decreased plasma glucose and insulin (5), decreased depletion of liver glycogen (6), increased plasma free fatty acids, free glycerol, and ketones (7), and decreased plasma triglycerides (8). Because fenfluramine exhibits concomitant anorectic and hypoglycemic effects, it has been suggested for possible use in the treatment of the obese, maturity onset, diabetic patient (9). Since the genetically obese mouse (ob/ob) is characterized by obesity associated with hyperglycemia, hyperinsulinemia, and insulin resistance similar to that observed in maturity onset diabetes, we investigated the effects of prolonged fenfluramine administration in this animal in comparison to the effects in mice made obese by gold thioglucose injections (GTG) and in nonobese mice.

Materials and methods. Animals used in the study were female, genetically obese mice of the C57BL/6J-ob strain (genotype, ob/ob), female mice of the C57BL/6J strain made hyperphagic and obese by a single ip injection of 600 mg/kg body weight GTG in distilled water at 9 weeks of age, and female nonobese C57BL/6J-ob (genotypes, +/+ and ob/+) mice (Jackson Laboratories, Bar Harbor, Maine). Animals were housed singly in wire-bottom cages in a light- (12-hr light/dark cycle) and temperature- (20 ± 2°) controlled room and were fed ground laboratory chow (Ralston Purina Company, St. Louis, Mo.) and water *ad libitum*. Food intake, water consumption, and body weight were recorded three times a week. At 15 weeks of age, animals were divided into groups of seven mice and given either tap water or a 60 mg/100 ml solution of fenfluramine · HCl (A. H. Robins Company, Richmond, Va.) in place of drinking water. After 4 weeks, the concentration of the fenfluramine solution was increased to 90 mg/100 ml. Both the fenfluramine solutions and the tap water were flavored with orange extract to minimize any differences in water intake due to taste.

At 2, 4, and 6 weeks after treatment was begun, nonfasted mice were anesthetized with 50% CO_2:50% O_2, and blood samples were collected from the retroorbital sinus in heparinized capillary tubes. Plasma glucose concentrations were determined by the method of Fales (10), plasma insulin concentrations by the method of Herbert *et al.* (11), and plasma triglyceride concentrations by an enzymatic method using a diagnostic reagent kit (Dow Diagnostics, Indianapolis, Ind.). After 8 weeks of treatment, all animals were fasted for 18 hr, killed by decapitation, and their blood was collected in heparinized tubes for final determinations of insulin, glucose, and triglycerides. A 500 mg sample of liver was quickly excised and digested in boiling 1 *N* NaOH, and the glycogen content was determined with anthrone reagent (12). Body composition was determined using the procedure of Leshner *et al.* (13). The results were analyzed by Student's *t* test for independent samples comparing fenfluramine-treated animals to

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untreated animals, and expressed as mean ± SE.

Results. During the first 4 weeks of treatment, the ob/ob mice received a significantly ($P < 0.05$) smaller dose of fenfluramine when expressed on a body weight basis, 52 ± 3 mg/kg/day compared to 71 ± 7 mg/kg/day for GTG mice and 72 ± 6 mg/kg/day for nonobese mice. This difference was attributable to the greater body weight of the ob/ob mice since they ingested a total of 2.1 ± 0.2 mg of fenfluramine per day, while the GTG and nonobese mice ingested 2.1 ± 0.2 and 1.6 ± 0.1 mg, respectively. During this period there were no significant differences in food consumption, water intake, and body weight between treated and untreated ob/ob mice. Both GTG and nonobese mice exhibited a significant ($P < 0.01$) and sustained reduction in water intake with a significant ($P < 0.05$), but transitory, reduction in food intake which paralleled the transitory reduction in body weight (Fig. 1). When the concentration of fenfluramine in the drinking water was increased to 90 mg/100 ml, all animals received an average daily dose of 104 ± 8 mg/kg. At this dose, all animals showed significant ($P < 0.05$) reductions in food and water intake. Body weights were also significantly ($P < 0.05$) reduced for all treated mice during the final 4 weeks when compared to untreated mice (Fig. 1). Fenfluramine treatment reduced body weight and prevented weight gain more effectively in the GTG mice than in the ob/ob mice and was least effective in nonobese mice.

The results of the biochemical determinations made during the final four weeks of treatment, when all animals received the same dose of fenfluramine, are presented in Table I. A striking effect of fenfluramine treatment was the reduction in plasma glucose concentrations in the ob/ob mice at both the sixth and eighth weeks of treatment under fed and fasted conditions, respectively. Fenfluramine significantly reduced plasma glucose concentrations in fed, but not in fasted, GTG mice. In contrast, there was no effect in the nonobese mice. Plasma insulin concentrations were reduced by fenfluramine treatment in the normally hyperinsulinemic ob/ob mice, but not to any significant degree in the GTG and nonobese mice. Fenfluramine treatment lowered plasma triglyceride concentrations in all treated animals, but only significantly in the GTG mice. The glucose and triglyceride values for fed animals (sixth week) presented in Table I are representative of the values obtained after 2 and 4 weeks of treatment at the lower dose, when the effects on food intake and body weight were much less. However, the significant reduction in plasma insulin concentrations in the ob/ob mice occurred only during the last weeks of

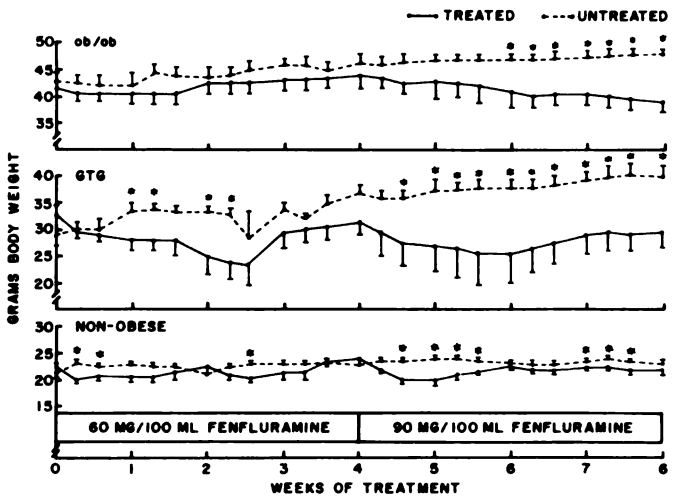

FIG. 1. Body weight changes in ob/ob, GTG, and nonobese mice treated with fenfluramine in drinking water. Bars represent SE and asterisks indicate significant ($P < 0.05$) differences between treated and untreated mice.

TABLE I. EFFECT OF FENFLURAMINE ON PLASMA CONCENTRATION OF GLUCOSE, INSULIN, AND TRIGLYCERIDES, AND ON LIVER GLYCOGEN IN MICE.

Parameter measured	Treatment week[a]	ob/ob		GTG		Nonobese	
		Treated	Untreated	Treated	Untreated	Treated	Untreated
Plasma glucose (mg/100 ml)	6	128 ±4 (6)[b]	285 ±27 (5)	122 ±17 (3)	171 ±3 (4)	110 ±6 (4)	110 ±8 (6)
		$P < 0.001$		$P < 0.05$		NS	
	8	90 ±3 (5)	195 ±5 (6)	97 ±21 (3)	137 ±8 (6)	82 ±14 (4)	89 ±11 (7)
		$P < 0.05$		NS		NS	
Plasma insulin (μU/ml)	6	36.7 ±1.4 (3)	63.5 ±6.6 (5)	11.5 ±1.4 (3)	18.8 ±3.3 (4)	11.9 ±3.9 (4)	27.2 ±6.6 (5)
		$P < 0.05$		NS		NS	
	8	66.5 ±7.8 (5)	90.0 ±5.1 (6)	34.8 ±2.6 (3)	26.2 ±3.9 (6)	32.3 ±3.7 (4)	44.9 ±1.8 (7)
		$P < 0.05$		NS		NS	
Plasma triglycerides (mg/100 ml)	6	219 ±39 (3)	251 ±38 (5)	78 ±5 (3)	154 ±4 (4)	80 ±10 (4)	125 ±36 (5)
		NS		$P < 0.05$		NS	
	8	80 ±14 (5)	99 ±22 (6)	72 ±7 (3)	114 ±14 (4)	62 ±2 (4)	99 ±26 (6)
		NS		$P < 0.05$		NS	
Liver glycogen (mg/g)	8	6.5 ±0.3 (5)	8.4 ±0.9 (6)	4.4 ±1.3 (3)	4.9 ±0.9 (6)	3.2 ±0.1 (5)	4.7 ±0.7 (7)
		NS		NS		NS	

[a] Six and eight treatment weeks were the second and fourth weeks after fenfluramine was increased to 90 mg/100 ml of drinking water.

[b] Values are mean ± SE; numbers in parentheses indicate number of determinations.

TABLE II. PERCENTAGE BODY COMPOSITION OF FENFLURAMINE-TREATED AND UNTREATED MICE.

	ob/ob		GTG		Nonobese	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
Water	39.1 ± 1.2 (5)[a]	38.8 ± 2.1 (6)	48.0 ± 1.0 (3)	46.1 ± 4.2 (6)	61.6 ± 1.9 (5)	68.0 ± 1.5 (7)
	NS		NS		$P < 0.05$	
Protein	10.0 ± 1.1 (5)	9.5 ± 0.3 (4)	14.7 ± 3.8 (3)	11.2 ± 1.1 (6)	8.8 ± 1.7 (5)	6.3 ± 1.0 (7)
	NS		NS		NS	
Fat	50.0 ± 8.3 (5)	43.7 ± 2.6 (6)	23.7 ± 1.9 (3)	27.8 ± 3.6 (6)	13.6 ± 1.2 (5)	10.1 ± 1.0 (7)
	NS		NS		$P < 0.05$	

[a] Values are mean ± SE; numbers in parentheses indicate number of determinations.

treatment when body weight was also significantly reduced. Liver glycogen concentrations were not significantly affected by drug treatment in any of the mice. The body composition data are given in Table II. Despite the significant reduction in body weight, the percentage of body fat was not significantly reduced by fenfluramine treatment.

Discussion. Body weight loss in man and other animals is correlated with the steady-state plasma concentration of fenfluramine and its active metabolite norfenfluramine, which represents a dynamic equilibrium between fenfluramine absorbed and that accumulated in body tissues (9). Le Douarec and Neveu (1) suggest that fenfluramine should be given at the time when food is eaten to be effective. In either case, administration of fenfluramine in the drinking water should be expected to promote the most effective reduction in food intake and body weight. Maximal effects of this compound were probably observed during the final 4 weeks of treatment when the animals were receiving 104 ± 8 mg/kg/day, which is five times the ED_{50} for ip fenfluramine in mice and 43% of the LD_{50} for orally administered

amine (1). However, it is possible treatment had continued, the effects of this higher dose would have died as it did for the lower dose during st 4 weeks of treatment (Fig. 1). On ıer hand, the tolerance observed dur- initial 4-week period could represent of this lower dose to produce an ve plasma fenfluramine concentra-)ur results fail to confirm reports (1, that tolerance to the anorectic action luramine does not develop readily.

orts of increased lipolysis (7) and de- d lipogenesis (15) in fenfluramine- l animals have led to the hypothesis nfluramine may contribute to weight ı direct effects on lipid metabolism as ; by reducing caloric intake. While a se in plasma triglyceride concentra- ıd the commonly associated increases fatty acids and ketones are often used ndication of lipolysis in fenfluramine- l animals, Garattini *et al.* (8) have that fenfluramine may reduce plasma erides by reducing the absorption of rom the gut. Furthermore, Dannen- *al.* (16) have shown that in the rat, amine reduces available energy by re- fat absorption, but that fenfluramine ɔt affect body composition. While the ion in plasma triglyceride concentra- ɔserved in the GTG mice (Table I) r may not be indicative of lipolysis, lure of 8 weeks of fenfluramine ad- ation to significantly reduce the per- ɛ of body fat in any of the treated Table II) leads us to the conclusion nfluramine does not enhance weight enhancing lipolysis in adipose tissue. sults also fail to confirm the observa- Duhault (6) that fenfluramine pre- he depletion of liver glycogen during (Table I).

observation *in vivo* (5, 17), that fen- ine reduced blood glucose and im- glucose tolerance, has been ex- l on the basis of *in vitro* observations nfluramine increased the uptake of ; by muscle (4), that this effect was ependent and significantly increased presence of insulin (18), and that the ıism of fenfluramine-induced glucose was different from that of insulin (19). Under the conditions described in our experiment, fenfluramine reduced blood glucose concentrations only when some degree of hyperglycemia was seen (Table I). The reduction in blood glucose observed in the ob/ob mice when the dose of fenfluramine was not sufficient to produce the other effects of this drug proves the direct hypoglycemic action of fenfluramine. Since the elevated insulin concentrations in the ob/ob mice were reduced in the fenfluramine-treated animals concomitantly with body weight, it was not possible to determine whether this reduction was a consequence of the reduction in body weight or the reduction in blood glucose levels. The report by Weisweiler and Schwandt (5) that fenfluramine improved glucose tolerance and lowered basal plasma insulin and glucose concentrations without any weight reduction suggests that fenfluramine may reverse the normal sequence of an initial weight loss leading to decreased insulin resistance and improved glucose tolerance. Further investigations in the ob/ob might clarify the means by which fenfluramine improves glucose tolerance.

Summary. Oral administration of fenfluramine for 8 weeks reduced food intake and body weight in ob/ob, GTG, and nonobese mice. The hyperglycemia and hyperinsulinemia present in the ob/ob mice were significantly reduced by this drug. Plasma glucose, but not insulin, was significantly reduced in GTG mice; neither was significantly reduced in nonobese mice. Fenfluramine reduced plasma triglycerides, but had no effect on liver glycogen concentration and did not reduce the percentage of body fat.

Partial support was provided by Grants No. AM-02911 and No. AM-00106 from NIH, USPHS, and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health. The fenfluramine · HCl was a gift of Dr. Oscar Klioze. We wish to thank Teresa Pasquine for performing the immunoreactive insulin assays.

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Received July 12, 1976. P.S.E.B.M. 1977, Vol. 15

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 121-124 (1977)

A Pressor Response Associated with Drinking in Rats (39617)

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Intracranial injections of angiotensin II (AII) produce drinking (3, 9, 12), a blood pressure increase (5, 11), and ADH release (6, 7, 10). In our work with rats that have chronically implanted ventricular cannulae and femoral artery catheters, we have observed two components in the pressor response to intraventricular (IVT) AII, one of which is associated with the act of drinking. The presence of this drinking-dependent blood pressure increase may produce inaccurate estimations of the pressor effect of IVT AII injections if it is not taken into account in experiments where conscious rats are tested with access to water. The following experiments were designed to investigate this component of the blood pressure increase and to identify the mechanism of the response.

Methods. The subjects were 40 male Sprague-Dawley rats plus 10 spontaneously hypertensive (SH) rats of the Okamoto strain weighing 300-400 g. All animals were implanted with lateral cerebral ventricular cannulae, chronic femoral or carotid artery catheters, and in nine rats a chronic femoral vein catheter. A 3-day interval was maintained between ventricular cannulation and testing. Vascular catheters were implanted on the day of testing under ether anesthesia. For mean blood pressure recording, the arterial catheter was connected to an extended piece of PE-50 tubing which led outside the cage to a Statham P23Gb blood pressure transducer. Heart rate was measured with a Beckman 7856B cardiotachometer that was triggered by pressure pulses. Blood pressure and heart rate were recorded on a Beckman R411 dynograph recorder. For intraventricular injections, the stylus in the guide cannula was removed and a 30-gauge injection cannula was inserted. The injection cannula sat flush with the end of the guide cannula and was connected by PE-10 tubing to a 25-μl Hamilton syringe mounted outside the home cage. All animals were tested in their home cages and were unanesthetized and unrestrained during testing.

Sixteen rats received two 500-ng AII IVT tests and 12 animals were given two 50-ng AII IVT injections. All central test solutions were dissolved in 5 μl of artificial CSF: Elliott's "B," Travenol Laboratories. For one of the tests, the animal was allowed to drink water for 15 min, and in the other, water was withheld during the 15-min testing session. Rats were randomly selected to begin the first AII test either with or without water. The two tests were separated by at least 1-hr intervals.

The magnitude of the drinking-dependent pressor response was also determined in five animals before and 10 min after peripheral blockade of the alpha-adrenergic system with 10 mg/kg of phentolamine, infused iv. The stimulus used to induce drinking before and after phentolamine infusions was 50 ng of AII IVT. At the completion of testing, animals were injected IVT with 5 μl of methylene blue dye. The brains were then removed to assure ventricular access of the injections. All data are reported as mean ± SE, and comparisons were made with a paired or Student's *t* test.

Results. The mean resting blood pressure for the normal rats was 104 ± 2 and 160 ± 4 mm Hg for the SH rats. An example of alternate IVT angiotensin tests in one animal with and without drinking in normal rats is shown in Fig. 1. With both 500- and 50-ng AII doses, the pressor response associated with drinking resulted in greater total pressor responses than when water was withheld during testing (Fig. 2). For 500 ng of AII, the pressor response with drinking was 30 ± 3 and 20 ± 2 mm Hg without access to water ($P < 0.001$). With 50 ng of AII, the pressor response with drinking was 26 ± 2 mm Hg, and 17 ± 2 mm Hg without ($P < 0.001$). Water intake averaged 8.8 ± 0.4 ml for the 500-ng AII tests and 4.8 ± 0.7 ml for the 50-ng AII tests. In four ani-

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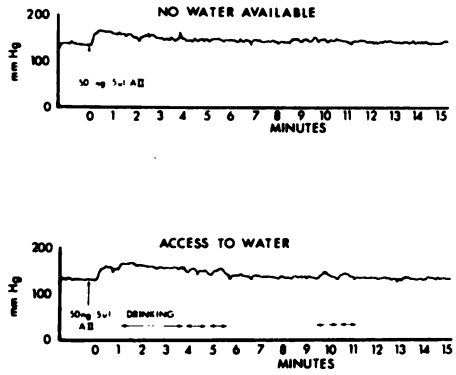

FIG. 1. Pressor responses without (top) and with (below) drinking allowed during the 15-min testing interval in Rat No. 809.

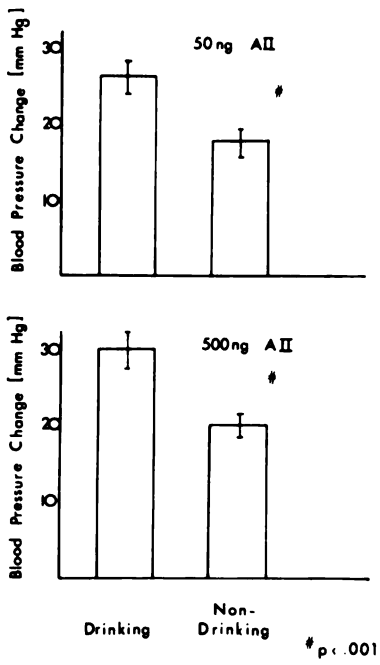

FIG. 2. Magnitude of blood pressure changes to AII IVT injections with and without drinking during the testing session; 500 ng of AII (n = 16); 50 ng of AII (n = 12). Paired Student's t test was used, drinking vs nondrinking. Values given as mean ± SE.

mals, drinking commenced after the initial pressor response had returned to baseline as in Fig. 3. In these cases, it was observed that the initial, drinking-independent response was associated with a bradycardia, while the drinking-dependent response was consistently coincident with an increase in heart

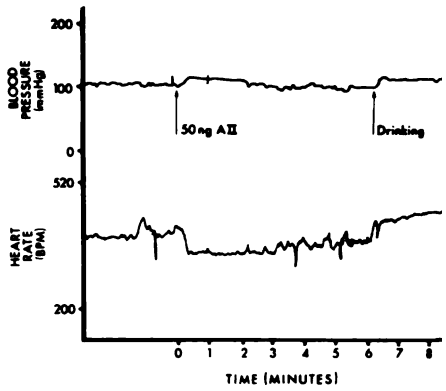

FIG. 3. Blood pressure and heart rate chang an IVT AII test in Rat No. 1128. Initial, drinl independent pressor response was associated wi bradycardia and drinking-dependent response wa sociated with a tachycardia.

rate (44 ± 8 min^{-1}). If drinking took p during the initial pressor response the a tional increase in blood pressure was usu associated with no change in heart rate though this was not quantitated. Ave latencies for the pressor and drinking sponses in these animals were 15 ± 2 an ± 18 sec, respectively. As Fig. 1 indica blood pressures remained elevated du the entire drinking bout. For 10 anir tested with and without drinking to 50(of AII IVT, the area under the press curves was 225 ± 14 and 155 ± 27 mm × min, respectively. This difference was nificant ($P < 0.05$, paired Student's t te indicating the pressor effect with drinl was a long-term effect. Ten animals tes with 50 ng of AII had an average pressur 185 ± 27 mm Hg × min with drinking 108 ± 15 mm Hg × min with no wa available ($P < 0.001$). The area under pressure curves of all animals was not eva ated because the chart speed was chan during the 15-min testing period in so cases. A pressor response during spor neous drinking was observed in 13 rats averaged 12 ± 3 mm Hg.

In 10 SH rats, the pressor response as ciated with drinking was identified se rately from the initial pressor response AII although these animals were not tes separately without access to water. pressor response associated with drinkin

rats averaged 25 ± 2 mm Hg. This was greater than the drinking-depend- ressure response seen in normotensive $P < 0.001$).

ur Sprague-Dawley rats were tested lrinking-dependent pressor effect to AII injections while drinking from a ing tube raised above the head, and ıdly from a dish of water on the floor of age in order to test the effect of head on on the pressor response. There was ıange in the drinking-dependent blood ure increase between these two tests. total pressor response with drinking the tube averaged 28 ± 1 and 29 ± 3 Hg with drinking from the dish. Like- two animals tested in an oxygenated ber still retained this short latency or response, indicated that hypoxia ot the initiating factor.

ntolamine, an alpha-adrenergic er, when infused iv abolished the pres- fect of drinking in all five rats tested. e phentolamine, the total blood pres- ncrease to 50 ng of AII IVT averaged 3 mm Hg and the animals drank 4.0 ± ıl. The drinking-associated pressor re- e averaged 9 ± 1 mm Hg. Intravenous oalmine decreased the resting blood ıre in these rats from 124 ± 2 to 77 ± Hg. After alpha-adrenergic blockade, ood pressure increase to AII was 19 ± Hg in these animals, and they drank : 0.9 ml. All animals drank in the hentolamine-testing session. How- the pressor response associated with ng was completely abolished by this ıent.

cussion. The various lines of evidence ave presented here have shown the ıce of a pressor response which is di- dependent on the act of drinking, and ently independent of how that drink- initiated. This response lasts only as s the drinking behavior and is depend- ı a functional peripheral sympathetic us system.

drinking-dependent response has een consistently observed in this labo- with drinking induced by intracranial hol injections, indicating that it is not ic to AII. In addition, we have ob- l the response with spontaneous drink- ing bouts. Therefore, it appears to be a blood pressure response associated with the act of drinking, no matter how the drinking is induced.

We have not been able to define the cause of this pressor response, but we can exclude certain possible sources for it. Since no change in the two-part pressor response to IVT AII injections was seen when the animal drank out of a dish, as opposed to the drinking tube, the drinking-associated pressor response is not due to the head position during drinking, eliciting a baroreceptor reflex. The possibility that during drinking, glottis closure produces a state of hypoxia, can be dismissed. Animals which were breathing 95% oxygen in an oxygenated chamber still showed the response. In addition, the blood pressure increase is observed a very short time after the start of drinking (10 s or less), too soon for hypoxia to become apparent. Volume loading by the water intake is also unlikely to be a cause of the pressor effect because of the fast latency of its appearance. The response is not observed with eating, showing that it is specifically related to drinking. This also suggests that general muscle activity is not responsible for the effect.

No significant difference was noted in the total pressor response to IVT AII injections after iv phentolamine treatment. This is due to an activation of both sympathetic pressor mechanisms and vasopressin release with IVT AII infusions (11). Following peripheral sympathetic blockade, the sympathetic component of this response is blocked. However, the cardiovascular response to circulating vasopressin is enhanced (6). This results in a total pressor response after sympathetic blockade which is not different from the normal response (6, 11). Of primary importance to this study, however, is the fact that the pressor response associated with drinking was abolished, indicating that this particular component is of sympathetic origin. This is consistent with our observations that the blood pressure increase with drinking occurs with a short latency and falls off immediately after the drinking stops. Tachycardia associated with the drinking-dependent pressor response was consistently observed and provided another indica-

tion of increased sympathetic activity. The greater increase in drinking-related pressor responses by SH rats as compared to normotensive animals may be due to increased sensitivity of the peripheral vascular system of SH rats to norepinephrine (8) and increased vascular responsiveness of these animals to all vasoconstrictor agents (2). Increased cardiovascular responsiveness in SH rats to sympathetic activation has been noted previously (1, 4).

The results of these experiments have significance relating to experimental procedures. The phenomenon may be quite widespread over several species such as sheep (Dr. E. Blaine, personal communication) and dogs (Drs. Reid and Ganong, personal communication). Investigators dealing simultaneously with drinking and pressor responses in unanesthetized animals and manipulations which will change one or both of these should be aware of the drinking-related pressor effect.

Summary. When angiotensin II is injected into the cerebral ventricles of the rat, drinking and a short latency pressor response are produced. We have found that the pressor response has two components. The first component is always associated with angiotensin injections. The second component, however, is associated with drinking behavior. If water is not available, the second pressor component is absent. We have investigated this drinking-associated pressor response and find that it is not only seen when angiotensin is the stimulus, but also in normal or carbachol-induced drinking. The effect is not due to head position during

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Responsiveness of Anestrous Labrador Bitches to GnRH (39618)

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eral studies have indicated that hypo-nic gonadotropin-releasing hormone H) can induce release of luteinizing one (LH) into the peripheral circula-n rats (1), hamsters (2), sheep (3), (4), and in primates including man (5, fectiveness of GnRH to release LH in nine, however, has not been reported. have been used extensively as a model l in biological research and particu-in medical research. Many breeds of ave relatively long periods of postpar-nestrus, and induction of ovulation regnancy could improve their breeding rmance. The present experiment was taken to evaluate the ability of anes-bitches to respond to varying levels of tic GnRH.

erials and methods. Twelve purebred dor bitches having a mean body t of 27.8 kg were assigned at random ir groups in equal numbers. All ani-have whelped at least once and were en 1 to 2 months following the last ing at the time of the experiment. al smears were checked from each ani-rior to the initiation of the experiment ure that all the animals were in anes-Each animal in all four groups was ed im with 1 ml of saline or 1 ml of containing varying amounts of GnRH tt Laboratories) as follows: group I, only; group II, 5 μg of GnRH; group μg of GnRH; and group IV, 50 μg of I. Blood samples were collected im-tely before the injection and at 15, , 90, 120, 240, and 360 min following jection of GnRH or saline from each l by jugular puncture. Blood samples allowed to clot and were stored at 4° ight, and the serum was separated by fugation and stored frozen until as-sayed. All samples were analyzed for serum LH content using a double-antibody radioimmunoassay for canine LH (7). A highly purified ovine pituitary LH preparation (LER-1056-C2) was used for iodination and a canine LH preparation (LER-1685-1) was used as the standard. The difference between pre- and post-treatment peak serum LH concentration (ΔLH) of each animal was used as an index of relative LH-releasing potency of different doses of GnRH. These data on ΔLH were then subjected to a linear regression analysis (8).

Results and discussion. All animals treated with GnRH showed an immediate increase in the serum levels of LH (Fig. 1). Animals treated with 5 and 25 μg of GnRH exhibited mean peak levels of 8.5 ± 3.2 and 18.7 ± 3.2 ng/ml, respectively, at 15-min following treatment. Serum LH levels returned to pretreatment levels by 2 hr following treatment in these groups of animals. Significant increases in serum LH concentrations within 15–30 min of treatment have been previously reported in pigs (9) and sheep (10).

Animals treated with 50 μg of GnRH showed a mean peak LH level of 39.7 ± 7.8 ng/ml at 30 min postinjection and returned to the pretreatment level after 4 hr. Similar observations have been made where higher levels of GnRH have resulted in both an increase in serum LH levels and duration of LH response (4). A peak LH level of 35.5 ng/ml during estrus has been reported in the beagle bitch (7). Saline-treated animals showed no prolonged elevated release of LH, although spontaneous releases of 6.1 and 3.8 ng/ml of LH were detected in two such animals. Treatment of anestrous bitches with varying doses of GnRH resulted in a linear response in serum LH increment (Fig. 2). The mean serum ΔLH responses to treatment with 5, 25, and 50 μg of GnRH were 6.5 ± 0.5, 17.2 ± 3.7, and 44.6 ± 7.8 ng/ml, respectively. Similar

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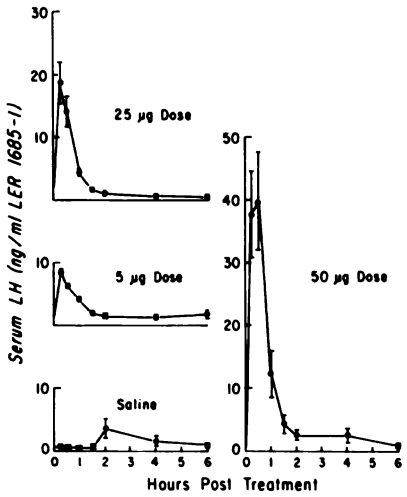

FIG. 1. Serum LH levels of anestrous Labrador bitches following injection of saline or varying doses of GnRH. Vertical bars indicate standard error of the mean.

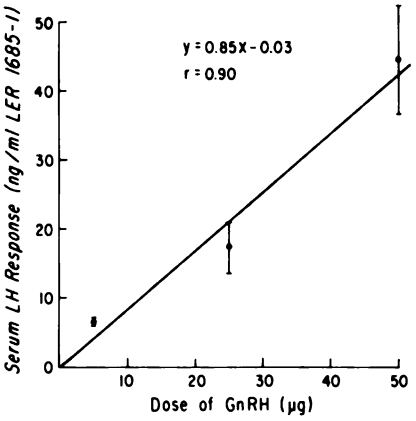

FIG. 2. Dose-response regression line for GnRH expressed as serum LH increment in anestrous Labrodor bitches. Vertical bars indicate standard error of the mean.

dose-response relationships with GnRH treatment in cattle have been reported earlier (4). The regression line predicting the ΔLH to a given dose of GnRH is given by the equation $Y = 0.85X - 0.03$ (where X = dose and Y = ΔLH). In the present study, a 50-μg dose of GnRH elicited a serum LH response in anestrous Labrador bitches which was similar to or higher than previously reported preovulatory surges of serum LH in the canine.

Summary. Serum LH concentrations of anestrous Labrador bitches treated with 5 or 25 μg of GnRH reached mean peak levels of 8.5 ± 0.5 and 18.7 ± 3.2 ng/ml, respectively, at 15 min following a single im injection. Animals treated with 50 μg of GnRH exhibited a peak LH concentration of 39.7 ± 7.8 ng/ml at 30 min post-treatment. A linear dose-response relationship was observed between the doses of 5 and 50 μg.

The authors wish to acknowledge the gift of purified canine LH (LER-1685-1) by Dr. L. Reichart, GnRH by Dr. R. Rippel (Abbott Laboratories), and ovine LH antiserum by Dr. G. D. Niswender. They also acknowledge the technical assistance of Dana McNaught and Michele Palmquist.

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Received June 1, 1976. P.S.E.B.M. 1977, Vol. 154.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 127-130 (1977)

ative Density of Sympathetic Nerve Terminals in the Canine Right Atrium[1] (39619)

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gh numerous studies have been :he regional differences in sympave supply to the heart of the dog, limitations have prevented quantimates of the density of nerve endn small portions of a given chammical assays for norepinephrine, ıical fluorescence, and radioactive hniques have furnished important ɔn, but definitive descriptions of stributions of sympathetic termistill lacking. As pointed out by s (1) in 1965, no samples from ions can be representative of the art, nor can estimates from the ɔn of the whole heart accurately tribution to selected regions. Even c question of the efferent innerva- SA nodal region remains open at ıt time. To obtain answers to the questions of specific innervation d regions of the right atrium, the ımber was examined under neur:t and extrinsically denervated ı radioactive tracer technique.

s. The studies employed the uptiated norepinephrine to map the ensities of sympathetic nerve ter-20 consecutive sections of the right ıcluding the interatrial septum. ıe 18 dogs used were subjected to ac denervation (2) approximately ior to the uptake study. The asepal procedures for denervation inreful dissection around the comımference of the superior vena C), transection of the azygous lissection around the right pulmoy. The dissection was continued rom the SVC across the superior the right and left atria, and the was removed from the complete circumference of the pulmonary artery. All nervous tissue was also removed between the roots of the pulmonary artery and the aorta. The ventrolateral cervical cardiac nerve was transected and all nervous tissue was removed from the region around the left superior pulmonary vein. Inotropic and chronotropic responses to bilateral stimulation of the thoracic vagosympathetic trunks, together with the left stellate ganglion, were performed immediately following surgery, and completeness of denervation was certified. These tests were repeated at the time of sacrifice in order to confirm the denervation.

Under phencyclidine HCl (2.0 mg/kg) and alpha chloralose (80 mg/kg) anesthesia, thoracotomized dogs were administered a slow injection containing 5 μCi of [1-^{3}H]norepinephrine (Amersham-Searle) via the SVC. After 20 min, the heart was excised and the right atrium and interatrial septum were dissected and consistently sectioned into 20 small portions of about 250-mg each, and immediately were immersed in cold saline (0.9% NaCl). The appendage was cut along its fold from its ventricular border to the SVC so that the entire chamber could be laid out flat. Natural landmarks such as the fossa ovalis, coronary sinus, and the crista terminalis allowed consistent separation into the 20 sections mapped in the upper portion of Fig. 1. The tip of the appendage is comprised of sections 1, 2, 3, 19, and 20. The interatrial septum was included in sections 13 and 14 and the SA node was always in section 5. All visible fat was trimmed from the sections, and the atrium was frozen before preparation for liquid scintillation counting.

Thawed sections of right atrium were weighed and ground in a Waring blendor with microattachment in 5 ml of 0.4 *N* perchloric acid. After grinding for 3 min, each section was centrifuged at 4000*g* for 10 min

restigation was supported by National Instiılth Grant Number HL 08682.

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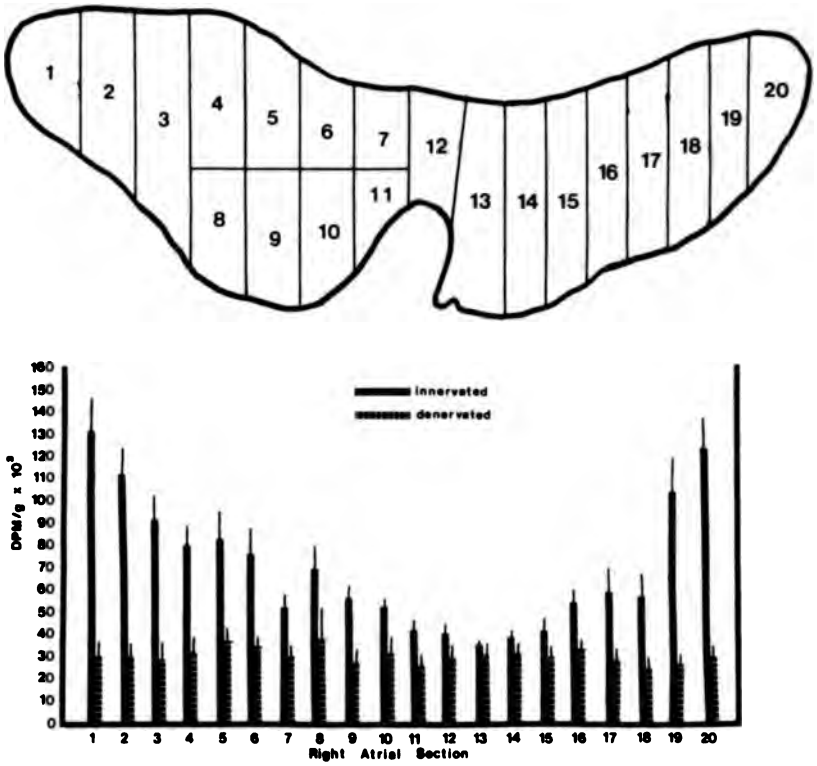

FIG. 1. The upper portion illustrates the sectional layout of the right atrium as observed from the endocardial surface. Sections 1 and 20 represent the apex of the appendage. The interatrial septum includes sections 13 and 14. The anterior free wall includes sections 4 through 11 and the posterior free wall sections 15 through 18. Lower portion illustrates the distribution of radioactivity (disintegrations per minute per gram $\times 10^3$) in innervated (solid bars) and totally denervated (striped bars) dog hearts following intravenous tritiated norepinephrine. Thin lines represent standard deviation.

in an International portable refrigerated centrifuge. Duplicate 1.0-ml samples of the supernatant were added to 9 ml of phase-combining solution (PCS, Amersham-Searle) which served as both a solubilizer and a scintillation cocktail. The samples were counted by liquid scintillation in a Searle-Analytic Isocap 300 System. The sample channels ratio method of quench correction was applied to each counting period and background radioactivity was taken into account. Uptake was then computed as disintegrations per minute of tritiated norepinephrine per gram wet weight of atrial tissue. All data were analyzed for statistical significance using one-way analysis of variance followed by a Scheffe test for multiple group comparisons.

Results. Figure 1 (lower portion) shows mean disintegrations per minute per gram of atrium (dpm/g) for each section of neurally intact atrium (solid bars) with standard errors of the mean (narrow bars). In general, a gradient of radioactivity became apparent from a high concentration in either tip of the appendage (section 1 = 130,000 dpm/g, section 20 = 122,000 dpm/g) to a low in the interatrial septum (section 13 at 35,000 dpm/g and section 14 at 37,000 dpm/g). Section 5, containing the SA node at 85,000 dpm/g was intermediate in general level of radioactivity. Statistical analysis showed section 1 to be significantly more radioactive than 7 ($P < 0.005$), 9-11 ($P < 0.005$), 12-15 ($P < 0.001$), 16-17 ($P < 0.025$), and 18 ($P < 0.01$). Section 2 was higher than 11 ($P < 0.05$), 13-14 ($P < 0.025$), and 15 ($P < 0.05$). Section 20 was higher

l ($P < 0.005$), 12 ($P < 0.01$), 13–14 .001), and 15 ($P < 0.005$). No other risons were statistically different.

re 1 also shows mean disintegrations ıute per gram for each segment of the ısly denervated heart (striped bars). :ral there was no gradient of radioac- ɔetween the regions of the totally, ically denervated right atrium. Statis- ıalysis revealed no significant differ- ɔetween any two sections. The aver- dioactivity of all 20 sections was dpm/g. Compared to 130,000 dpm/ :ction 1 of the innervated atrium, this :nts a reduction to 21.5%.

ussion. Other studies employing dif- techniques have investigated the re- differences in sympathetic supply to g heart and have reported varying and conflicting conclusions (3–7). :er, the concept of high levels of ra- vity in the appendage, implying a re- : dense sympathetic nerve terminals, ɔport in the literature. Dahlstrom (4) a "large number" of sympathetic ter- there although she offered no quanti- description. The significance of this :nsity may be functional in regulating e of increased contractility required the stress of acute distention. This esis requires further study.

ɔchemical fluorescence techniques lemonstrated marked variability in ıtion of adrenergic innervation to ssues. Fluorescence patterns have in- a rich supply to atrial tissues (as :ed to ventricular) with a particularly ıupply to SA nodal regions (4). The to Purkinje cells, on the other hand, to be very sparse, with many such ceiving no adrenergic innervation at ally, these histochemical techniques early differentiated rich distribution nergic nerves to "upper regions" of ıt atria from that to areas near the de which are relatively poorly sup-), 4). Considerable functional impor- :ould be imputed to a particularly listribution of adrenergic fibers to the zed internodal conduction pathways, ther the fluorescence nor the present l norepinephrine uptake studies lend :e to that mechanism.

On the other hand, there is conflicting evidence concerning the densities of terminals within and around the SA node. Shindler (6) reported a higher concentration of endogenous norepinephrine in this location than in the remainder of the right atrium. Serrano (5), however, concluded from measurements of endogenous norepinephrine that the SA node did not have higher levels. Shindler compared the SA node to atrium exclusive of the appendage, and in light of the present data, this would account for the difference. However, extrapolation from SA nodal region concentration does not necessarily prove that the SA node itself is not well supplied with sympathetic nerves, because it is a relatively small portion of the total tissue sample. Based upon calculations of SA nodal volume of 5.0 mm^3 (8), the SA node represents only about 5% of the volume of the total sample (approximately 100 mm^3).

The result of a statistically significant peak of radioactivity in the right atrial appendage attests to the sensitivity of [^{3}H]norepinephrine uptake as a quantitative evaluation of sympathetic nerve terminal density. The results uphold the expectation that this technique is a useful gauge for the status of denervation. The chronically denervated sections were, on the average, 21.5% of the maximum levels observed in the innervated atria. The fact that uptake was not reduced to zero after denervation was to be expected on the basis of known extraneuronal uptake mechanisms (9). The disappearance from denervated atria of the gradient of radioactivity seen in neurally intact atria confirms the assumption that the gradient was produced by the presence of sympathetic nerves, and that lack of that gradient indicates sympathetic denervation.

Summary. Relative densities of sympathetic nerve terminals in 20 consecutive sections of both normally innervated ($n = 10$) and chronically denervated ($n = 8$) dog right atria were determined by comparative uptakes of [^{3}H]norepinephrine. Results of analysis of neurally intact atria display a consistent gradient of [^{3}H]norepinephrine with the highest concentration (130,000 dpm/g) in the tip of the atrial appendage and the lowest (30,000 dpm/g) in the intera-

trial septum. The region containing the SA node was not distinguished by significantly high uptake. Atria of chronically denervated hearts had a markedly diminished [^{3}H]norepinephrine uptake (average of 28,000 dpm/g). No gradient of radioactivity between sections was present in the denervated atria. These results indicate that (1) the canine right atrium has marked concentrations of sympathetic terminals in the appendage, (2) the technique of [^{3}H]-norepinephrine uptake gives a quantitative evaluation of sympathetic nerve terminal density, and (3) the technique allows for evaluation of the status of cardiac denervation.

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Received May 20, 1976. P.S.E.B.M. 1977, Vol. 154

S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 131-133 (1977)

nthesis of Fatty Acids in Perfused Hearts of Rats Fed a Fat-Supplemented or a Fat-Free Diet[1] (39620)

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heart is dependent on lipid metabo-
or structural components as well as for
of its caloric requirements. Fatty acids
ese needs are furnished to the heart
absorbed dietary lipids, from meta-
products of these absorbed lipids, and
lipids synthesized or interconverted
other components by the heart itself.
ypes of fatty acids biosynthesized and
ids into which these fatty acids are
orated are, therefore, of interest. As
of a continuing investigation of lipid
olism in the heart, we have made such
s in the rat heart perfused with labeled
e or butyrate. Because dietary factors
nce many aspects of lipid metabolism,
ve used hearts from rats fed a fat-free
s well as from rats fed the same diet
mented with corn oil.

hods and materials. A purified diet
ining 66% sucrose, 24% casein, 3%
als, 5% corn oil, and complete vi-
mix was used as the fat-supplemented
For the fat-free diet, the corn oil was
d and an equal weight of sucrose was
tuted. Male, weanling Sprague-Daw-
ts were maintained on either of these
or 3 to 6 months before the perfusion
s were done. Perfusion with a recircu-
system was done as reported previ-
(1).

thods for the extraction of total lipids
paration into lipid classes and for the
on of fatty acids after hydrolysis of
lipids have been described (2). Lipid
s were separated by thin-layer chro-
graphy using silica gel HF_{254} (E.
, West Germany) and the solvent sys-
petroleum ether–diethyl ether–glacial
acid (80:20:1, by volume). Bands of
gel containing the individual compo-
were immediately scraped from the
and the lipids were eluted according
technique of Biezenski (3). The re-
covery of radioactivity from the plates was greater than 85% in all cases and usually greater than 90% of the amount applied to the plates. Gas–liquid chromatography and gas–liquid radiochromatography were done as described previously (4).

Perfusion of [1-^{14}C]sodium acetate and of [1-^{14}C]sodium butyrate was done using a concentration of 5 m*M* and 20 μCi of radioactivity in 25 ml of perfusion medium (oxygenated Krebs–Henseleit bicarbonate buffer, pH 7.4, containing glucose at a concentration of 5 m*M*). Perfusion was carried on for 2 hr in an apparatus similar to that described by Morgan *et al.* (5).

Results. Body and heart weights of rats fed the fat-free diet were lower than those of rats fed the fat-supplemented diet (body weight averages: 248 vs 344 g; heart weight averages: 1.74 vs 2.03 g). Incorporation of [1-^{14}C]acetate into fatty acids of perfused hearts was not significantly different from the value of 0.2 to 0.3% of the added substrate reported previously (1), and there was no difference between hearts of rats fed the different diets. Incorporation of [1-^{14}C]butyrate averaged 0.44 ± 0.12 in the rats fed the supplemented diet versus 0.81 ± 0.13% of added substrate in hearts of rats fed the fat-free diet. This difference was of borderline statistical significance with a *P* value of 0.07.

The distribution of radioactivity in the various lipid classes of hearts of rats fed the fat-supplemented diet is shown in Table 1. A greater percentage of the ^{14}C incorporated into total lipids was incorporated into phospholipids when [^{14}C]acetate was the substrate than when [^{14}C]butyrate was the substrate. ^{14}C from the latter was incorporated into all other lipid fractions to a greater extent than [^{14}C]acetate. However, the differences in values for cholesteryl esters and for unesterified fatty acids were not statistically significant.

With [^{14}C]acetate as substrate, no differ-

ported by USPHS Grant No. AM-06483.

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TABLE I. DISTRIBUTION OF ^{14}C IN LIPIDS OF HEARTS OF RATS FED A FAT-SUPPLEMENTED DIET AND PERFUSED WITH [1-^{14}C]ACETATE OR [1-^{14}C]BUTYRATE

	Total ^{14}C (%)				
Substrate	Phospholipids	Cholesterol plus diglycerides	Unesterified fatty acids	Triglycerides	Cholesteryl esters
[1-^{14}C]Acetate (3)[a]	74.4 ± 3.7[b]	1.6 ± 0.17	5.1 ± 1.5	15.1 ± 3.8	0.4 ± 0.12
[1-^{14}C]Butyrate (6)	52.3 ± 1.5	4.3 ± 0.35	13.0 ± 2.7	23.4 ± 1.7	1.0 ± 0.27
P value	<0.01	0.03	n.s.[c]	0.05	n.s.

[a] (n) = Number of animals.
[b] Mean ± SE.
[c] n.s. = Not significant.

TABLE II. DISTRIBUTION OF ^{14}C IN FATTY ACIDS OF HEARTS OF RATS FED A FAT-SUPPLEMENTED DIET AND PERFUSED WITH [1-^{14}C]ACETATE OR [1-^{14}C]BUTYRATE

	^{14}C in total fatty acids (%)							
Substrate	<14:0	14:0	16:0 16:1	18:0 18:1	18:3 20:1	20:2	20:4	>20:4
[1-^{14}C]Acetate (3)[a]	12.1 ± 0.35[b]	9.7 ± 1.6	32.3 ± 5.1	27.2 ± 0.96	3.1 ± 0.23	2.4 ± 0.23	1.9 ± 0.40	2.4 ± 0.23
[1-^{14}C]Butyrate (6)	31.8 ± 4.0	11.6 ± 0.67	20.8 ± 1.8	25.0 ± 1.6	2.3 ± 0.71	3.6 ± 1.2	0.7 ± 0.24	1.6 ± 0.55
P value	0.01	n.s.[c]	0.04	n.s.	n.s.	n.s.	0.04	n.s.

[a] (n) = Number of animals.
[b] Mean ± SE.
[c] n.s. = Not significant.

ences were observed between rats fed the supplemented and the fat-free diets with respect to relative incorporation of ^{14}C into the different lipid classes. With [^{14}C]-butyrate as substrate there was more ^{14}C incorporated into phospholipids of hearts of rats fed the fat-free than in those fed the fat-supplemented diet (62.5 ± 2.8 vs 52.3 ± 1.5% of total ^{14}C incorporated; P = 0.01). Although no other differences were statistically significant, less ^{14}C was present in the triglycerides of the deficient compared to those of the supplemented group.

The relative distribution of the incorporated ^{14}C into the individual fatty acids of perfused hearts of rats fed the supplemented diet is given in Table II. Use of [^{14}C]-butyrate as substrate resulted in more ^{14}C incorporation into shorter chain fatty acids (<14:0)[2] and less ^{14}C incorporation into palmitic acid (16:0) and into arachidonic acid (20:4) than use of [^{14}C]acetate. In the combined 16:0, 16:1 fraction nearly all the [^{14}C]acetate was in 16:0, while in the combined 18:0, 18:1 fraction the activity was divided about equally between the saturated acid and the monoene.

[2] Number of carbon atoms in chain: number of double bonds.

With either [^{14}C]acetate or [^{14}C]butyrate as substrate there were no statistically significant differences between rats fed the fat-supplemented and those fed the fat-free diet in the distribution of ^{14}C into individual fatty acids.

Biochemical signs of deficiency were evident in the hearts of the rats fed the fat-deficient diets. About 20% of the phospholipid and about 7% of the triglyceride fatty acids were 20:3w9, the fatty acid which accumulates in essential fatty acid deficiency. No measurable amount of this fatty acid was present in the heart lipids of the rats fed the fat-supplemented diet.

Discussion. The main difference in the incorporation of these two ^{14}C-labeled substrates into perfused heart lipids was the relatively greater incorporation of [^{14}C]-acetate into phospholipids and the greater incorporation of [^{14}C]butyrate into triglycerides. The use of [^{14}C]butyrate as substrate resulted in less ^{14}C in palmitic and in arachidonic acids while [^{14}C]butyrate yielded more ^{14}C-shorter chain acids than did [^{14}C]acetate. It is known that in the perfused heart of the rat, [^{14}C]acetate can be incorporated into fatty acids both by the *de novo* and elongation pathways (1). The manner by which ^{14}C from butyrate enters

chain fatty acids is not known, but ıably the butyrate may be oxidized to CoA units which then can form fatty or butyrate itself may be elongated to chain fatty acids.

›genesis in some tissues is affected by ıctors as diet and conditions such as ɛs. Whereat and Orishimo observed e heart mitochondrial system for syn- ɔf fatty acids was not affected by fast- d by diabetes (6). It is not known ɛr cytosol synthetic activity in heart is subject to the same regulations t in other tissues. Likewise, the effect ɛntial fatty acid deficiency on fatty nthesis by heart mitochondria or mi- ıes apparently has not been investi- (7). Our studies with perfused heart e that the feeding of a fat-free diet, ı it resulted in a decrease in arachi- ıcid and an increase in 5,8,11-eicosa- c acid in the heart, apparently did ɛct the metabolism of the [^{14}C]acetate ɛd for 2 hr. In the case of the butyr- ›wever, there was increased incorpo- in hearts of rats fed the fat-free diet, lt which is similar to that seen with fatty acid-synthesizing systems of or- ıch as the liver. In rats fed the fat- mented diets, more of the ^{14}C incor- d into lipids was in phospholipids ›as observed in rats fed the fat-free lowever, there was no effect on the ual fatty acids synthesized. It was ob- that the hearts of rats fed the fat-free d a weaker contraction and a slightly rate of contraction than did hearts ıts fed the fat-supplemented diet. Al- this is not a precise quantitative ob- ɔn, it is interesting in view of the finding reported by Caster and Ahn (8) of a notch in the QRS complex of the electrocardiograms of rats fed diets deficient in essential fatty acids. The notching was prevented by the addition of linoleate, linolenate, or arachidonate to the diet.

Summary. Incorporation of ^{14}C from [1-^{14}C]butyrate into lipids of the perfused rat heart was slightly greater than that from [1-^{14}C]acetate. Of the ^{14}C incorporated into total lipids, a greater proportion was in phospholipids when [^{14}C]acetate was the substrate than when [^{14}C]butyrate was used as substrate. Perfusion with [^{14}C]butyrate resulted in more ^{14}C incorporation into shorter chain fatty acids and less incorporation into palmitic and arachidonic acids than with [^{14}C]acetate. Feeding a fat-free diet did not affect the metabolism of acetate in the perfused heart. However, it did increase the relative incorporation of ^{14}C from butyrate into phospholipids as well as the total ^{14}C incorporation into total lipids of perfused hearts of rats compared to those fed a fat-supplemented diet.

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Received July 19, 1976. P.S.E.B.M. 1977, Vol. 154.

Tissue Distribution of [1-^{14}C]Cholylglycine i Bile Duct Liga

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Plasma disappearance of substances taken up largely by the liver, such as bromosulfophthalein (BSP) (1-3) or indocyanine green (4, 5), has been used widely as a test for liver function. Recently, the plasma disappearance of a bile acid, cholylglycine, has been proposed as a test for hepatic injury (6, 7). This bile acid clearance is based on the assumption that the plasma disappearance of cholylglycine may be equated with hepatic uptake. To test this assumption, we have defined the tissue distribution of intravenously injected cholylglycine in rats and hamsters; we have used animals with an acute bile fistula, as well as animals with a bile duct obstruction.

Materials and methods. Experimental design. A mixture of [1-^{14}C]cholylglycine and [^{99}Tc]albumin was injected intravenously as a bolus. Ten minutes later, animals were killed and tissues were removed for determination of radioactivity. The ^{99}Tc was used to estimate the amount of blood contaminating each tissue sample. The sampling time of 10 min was chosen since this time interval corresponds to about three half-lives for plasma disappearance of cholylglycine (in man) (8) and was judged to be a sufficiently long interval for nearly complete tissue uptake to occur.

Chemicals. [1-^{14}C]Cholylglycine (sp act 51.7 mCi/mmole) was purchased from Amersham/Searle (Arlington Heights, Ill.) and had a radiopurity greater than 96% by zonal scanning (9) using a thin-layer system for bile acid conjugates (10). [^{99}Tc]Albumin was prepared using a commercial kit (New England Nuclear Radiopharmaceutical Division, North Billerica, Mass.). A solution

[1] Supported in part by NIH Grant No. AM 16770. Dr. Ng was an NIH Postdoctoral Trainee under Grant No. AM 0599.

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ients Division, Irvine, Calif.). Samples then kept until the ^{99}Tc radioactivity decayed for at least 10 half-life times. ples were then oxidized using a wet oustion technique (11), and ^{14}C radioity was determined by liquid scintillaspectroscopy.

order to validate the wet combustion edure used for tissues, samples of some were digested in 10% KOH in order ermit liquid scintillation counting. stion in KOH gave higher radioactivity veral tissues than the perchloric acidzed wet oxidation technique, but difces were sufficiently small that the wet tion procedure was judged to afford titative recovery of radioactivity. In ase, for major organs, results obtained both methods were in good agree-

order to determine radioactivity present carcass, the carcass was suspended in KOH with a homogeneous solution ring in several days. Aliquots were , bleached with 30% hydrogen perox- 80° for 1 hr, and radioactivity was mined in a toluene-based scintillation re containing a solubilizer.

culations. From the ratio of ^{14}C to ^{99}Tc blood sample, the true tissue radioacwas calculated. Weights of individual s were determined on a number of ls (Table I) and were used to calculate activity present in the entire tissue.

preliminary experiments it was found he [^{99}Tc]albumin contained free ^{99}Tc was taken up excessively by some s (liver, spleen, kidneys), resulting in a y high value for entrapped blood. To ct for this, each batch of [^{99}Tc]albumin ixed with [^{125}I]albumin (Mallinckrodt ar, Mallinckrodt, Inc., St. Louis, , and the mixture was injected into rats amsters. The ratio of ^{99}Tc to ^{125}I in tissue was obtained 10 min later and sed to correct observed ^{99}Tc counts.

tistical analyses were done using the nt's *t* test.

ults. Healthy bile fistula animals. The distribution of the intravenously adtered [1-^{14}C]cholylglycine in healthy stula and bile duct-obstructed rats and ters is shown in Tables II and III, revely. It is obvious that in the healthy bile fistula animals, the liver is the organ responsible for the removal and excretion of serum bile acid. More than 50% of the injected ^{14}C radioactivity was excreted into the fistua bile within 10 min, while less than 1% remained in the circulation. The other organs did not pick up any appreciable amount of radioactivity.

TABLE I. ORGANS AND TISSUE WEIGHT OF GOLDEN SYRIAN HAMSTERS AND SPRAGUE-DAWLEY RATS.[a]

Organs	Hamster	Rat
Blood	7.32 ± 0.04[b]	7.29 ± 0.32[b]
Enterohepatic		
Liver	4.36 ± 0.38	4.08 ± 0.16
Stomach	0.77 ± 0.03	0.46 ± 0.02
Small intestine	1.44 ± 0.02	2.03 ± 0.11
Colon	0.86 ± 0.01	0.47 ± 0.01
Cecum	0.56 ± 0.01	0.29 ± 0.02
Cardiovascular-renal		
Heart	0.33 ± 0.01	0.30 ± 0.01
Lungs	0.60 ± 0.07	0.61 ± 0.02
Kidneys	1.07 ± 0.22	0.76 ± 0.01
Others		
Pancreas	0.13 ± 0.01	0.20 ± 0.03
Spleen	0.14 ± 0.02	0.33 ± 0.03
Testes	2.89 ± 0.10	1.15 ± 0.07
Uterus	0.39 ± 0.02	–
Brain	0.74 ± 0.02	0.64 ± 0.03

[a] Weights are expressed as g/100 g animal ± SE. Averages are of four animals each.
[b] ml/100 g animal.

Bile duct-obstructed animals. In the bile duct-obstructed animals, much more radioactivity was retained in the blood. In the bile duct-obstructed rats, all the major organs, except the liver, testes, brain, and the upper small intestine, contained a significantly larger amount of injected ^{14}C radioactivity when compared with the corresponding organs of the bile fistula rats (Table II). In the bile duct-obstructed hamsters, a similar increase in the uptake of ^{14}C radioactivity was seen in most major organs with the exceptions of the pancreas, the upper small intestine, and the colon. Uptake of ^{14}C-label by the liver was significantly greater in the bile duct-obstructed hamsters than in the bile fistula hamsters ($P < 0.05$), but there was no significant difference in liver uptake in the two groups of rats (Table II).

The total recovery of injected ^{14}C radioactivity from the bile fistula animals was between 72-78%. The ^{14}C radioactivities contained in the bones, skin, and fat were not included. Because in the bile duct ob-

TABLE II. TISSUE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED [1-^{14}C]CHOLYLGLYCINE AT 10 MIN IN RATS.[a]

Organs	Healthy acute bile fistula rats (N = 9)	4-Day biliary obstructed rats (N = 9)	
Blood	0.89 ± 0.1	19.69 ± 0.73	
Enterohepatic			
Liver[b]	12.48 ± 1.40	10.07 ± 1.02	N.S.
Bile	57.68 ± 2.76	–	
Stomach	0.17 ± 0.02	0.04 ± 0.03	
Upper intestine	2.21 ± 0.44	1.30 ± 0.5	N.S.
Lower intestine	0.71 ± 0.08	1.35 ± 0.7	
Colon	0.17 ± 0.03	0.34 ± 0.02	
Cecum	0.13 ± 0.02	0.24 ± 0.01	
Cardiovascular-renal			
Heart	0.02 ± 0.01	0.12 ± 0.01	
Lungs	0.10 ± 0.01	0.66 ± 0.03	
Kidneys	0.49 ± 0.04	2.75 ± 0.20	
Others			
Pancreas	0.09 ± 0.01	0.23 ± 0.02	
Spleen	0.05 ± 0.01	0.11 ± 0.01	
Genitals	0.20 ± 0.02	0.24 ± 0.02	N.S.
Muscle[c]	4.26 ± 0.62	14.96 ± 0.78	
Brain	0.02 ± 0.01	0.04 ± 0.01	N.S.
Total recovery	78.21 ± 2.56	51.28 ± 1.35	

[a] All values are significant at 0.1% level ($P < 0.001$), except when denoted N.S. (nonsignificant). Results a expressed as percentage dose administered per organ (mean ± SE).
[b] Liver includes intrahepatic biliary tract.
[c] Muscle mass is taken to account for 40% of the total body weight of the animal (20).

TABLE III. TISSUE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED [1-^{14}C]CHOLYLGLYCINE AT 10 MIN IN HAMSTERS.[a]

Organs	Healthy acute bile fistula hamsters (N = 5)	4-Day biliary-obstructed hamsters (N = 7)	P
Blood	0.41 ± 0.05	16.43 ± 1.17	<0.001
Enterohepatic			
Liver[a]	12.79 ± 1.53	19.80 ± 2.40	<0.05
Gallbladder	0.07 ± 0.02	0.1 ± 0.04	<0.001
Bile	55.29 ± 2.54	–	
Stomach	0.14 ± 0.01	0.45 ± 0.04	<0.001
Upper intestine	0.56 ± 0.13	0.82 ± 0.07	N.S.
Lower intestine	0.21 ± 0.02	0.91 ± 0.13	<0.001
Colon	0.33 ± 0.05	0.45 ± 0.04	N.S.
Cecum	0.09 ± 0.01	0.29 ± 0.04	<0.001
Cardiovascular-renal			
Heart	0.01 ± 0.01	0.11 ± 0.01	<0.001
Lungs	0.07 ± 0.01	0.43 ± 0.04	<0.001
Kidneys	0.18 ± 0.01	0.96 ± 0.06	<0.001
Others			
Pancreas	0.05 ± 0.01	0.06 ± 0.01	N.S.
Spleen	0.03 ± 0.01	0.02 ± 0.01	<0.001
Genitals	0.11 ± 0.02	0.42 ± 0.05	<0.001
Muscle	2.28 ± 0.22	11.48 ± 0.93	<0.001
Brain	0.01 ± 0.01	0.02 ± 0.01	<0.05
Total recovery	72.30 ± 3.68	51.78 ± 0.96	<0.001

[a] Results are expressed as percentage dose administered per organ (mean ± SE).
[b] Liver includes intrahepatic biliary tract.

structed hamsters the recovery, as calculated, was only slightly better than 50%, the carcass of several such animals were dissolved in 10% KOH solution. The total recovery thus obtained ranged from 91–95%. Similar recovery studies were carried out in two bile fistula hamsters; total recovery was 90 and 94%.

It is of interest to note that in bile duct-obstructed hamsters, about 10% of the injected ^{14}C label resided in the skin.

Discussion. In the healthy rat and hamster, radioactivity was taken up entirely by the liver and promptly excreted into bile. The hepatic capacity for bile acid uptake is known to be extraordinarily high (12, 13), but it is not known whether the absence of appreciable uptake by other tissues is because bile acids are largely albumin bound, or whether the membrane of the hepatocyte has a particular affinity for the bile acid molecule, or both. Single pass hepatic clearance of cholylglycine is nearly complete, based on studies in the dog (14), so that hepatic removal appears to be blood flow limited.

In the bile duct-obstructed rat and hamster, there was striking plasma retention. Tissue concentrations were significantly higher, but there was no particular organ that showed an especially enhanced uptake.

All in all, these data support the report of Bergstrom *et al.* (15) that in the healthy rat, bile acids are largely localized to the enterohepatic circulation. In bile duct obstruction, it is now recognized that there is extensive sulfation of bile acids (16, 17) promoting renal excretion. The anatomical distribution of injected bile acids in the chronically jaundiced rat was recently reported by Hanitkevych *et al.* (18) who observed that 2 weeks after bile duct ligation, bile acids were present largely in plasma and kidney and not in other tissues. The data thus imply that in healthy man, plasma disappearance of injected cholylglycine may be equated with hepatic uptake. Nonetheless, this point cannot be tested directly in man until a bile acid tagged with a gamma-emitting isotope is synthesized.

Recently Klaassen (19) examined the tissue distribution of intravenously administered bromosulfophthalein in the rat. He obtained tissues at 10 min as in our studies and corrected for trapped plasma. His data cannot be compared directly with ours, but he found a greater fraction of bromosulfophthalein was present in the kidney than we observed for cholylglycine.

We would like to thank Mr. Curtis Grabau for the preparation and care of the animals.

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Received April 30, 1976. P.S.E.B.M. 1977, Vol. 154.

Relative Ability of Mitogens to Stimulate Pr and to Induce Suppression of the *in*

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T lymphocyte mitogens are excellent inducers of suppressor cell activity in mouse lymphoid cell cultures (1–4). An array of lymphokines, including interferon, is produced by lymphocyte cultures that have been stimulated by these mitogens (5–7). In the mouse system, mitogen-induced (mitogen-type) interferon is antigenically different from virus-induced (virus-type) interferon (7), and may be identical or related to antigen-induced (antigen-type) interferon (8). It is becoming clear that, depending upon the inducer and the stimulated cell, there are several types of interferons that can be produced by mammalian cells. The following is defined for operational purposes:

1. *Virus-induced (virus-type) interferon.* This term is used for interferon produced in lymphoid or nonlymphoid cells following stimulation by virus or double-stranded RNA. This interferon is sometimes called type I interferon in the murine system.

2. *Mitogen-induced (mitogen-type) interferon.* This term is used for interferon produced in lymphoid cells following stimulation by T lymphocyte mitogens. This interferon is antigenically distinct from virus-induced (virus-type) interferon.

3. *Antigen-induced (antigen-type) interferon.* This term is used for interferon produced in antigen-primed lymphoid cells upon second exposure to the specific antigen. This interferon is also antigenically distinct from virus-induced (virus-type) interferon, and is sometimes called type II interferon in the murine system. It is tentatively felt that mitogen- and antigen-type interferons are the same substances in the murine system since no evidence to the contrary is presently available.

We are interested in the possible role of mitogen-induced interferon in the mediation of mitogen-induced suppressor cell activity. Several T cell mitogens are compared

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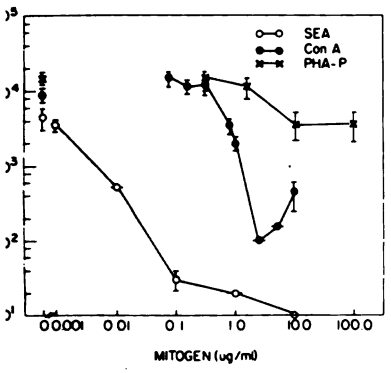
tures consisted of 1 ml of 1.5×10^7 ells and 3×10^6 SRBC. All PFC :s were determined at Day 5. Cell :s were determined by trypan blue usion. Direct PFC assays were per- on microscope slides as previously d (12). A single lot (C640521) of f serum, obtained from Gibco Diag- Grand Island, New York, was used out the study. All results are ex- as the average of duplicate cultures.

en induction of interferon. Mouse ell cultures were prepared exactly as d above for *in vitro* PFC response or the absence of SRBC. Various rations of the mitogens were added ultures and the cells were routinely d for 48 hr under the above de- conditions (9). Supernatant fluids tained by centrifugation of the har- ultures at 1000 rpm in an RC-3 centrifuge at 7°. These fluids were on the day of harvest for mitogen- interferon activity in mouse L cells ght modification of the method of n of cytotopathic effect of vesicular is virus (100 $TCID_{50}$/challenge 3).

s and discussion. The relative abili- various concentrations of Con A, and SEA to inhibit the PFC re- vhen added to cultures at the same ntigen are shown in Fig. 1. Coeffi- cients of variation for duplicate determinations were generally less than 20%. SEA was the most effective inhibitor, 0.01 μg/ml resulting in 88% inhibition of the PFC response, while 0.1 μg/ml and greater concentrations caused >99% inhibition of the anti-SRBC PFC response. Con A was the second-most effective inhibitor, with 1.25 μg/ml inhibiting the PFC response by 78%. Concentrations of 2.5 μg/ml or greater resulted in >90% inhibition of the anti-SRBC PFC response. Interestingly, 2.5 μg/ml of Con A was more inhibitory than 5 and 10 μg. This pattern of inhibition was observed in repeated experiments. Con A was nontoxic to spleen cells up to 2.5 μg/ml. Slight toxicity was observed at 5 and 10 μg of Con A. PHA-P was the least effective inhibitor of the PFC response; 10 μg/ml was required for 76% inhibition.

Representative data demonstrating the relative abilities of Con A, PHA-P, and SEA to stimulate mouse spleen cell cultures to produce mitogen-type interferon are presented in Fig. 2. Coefficients of variation for duplicate determinations were generally less than 25%. The interferon assays were carried out in cultures separate from those used for PFC responses, because of the differences in incubation times of the two systems. The data in Figs. 1 and 2 are evalu-



. The suppressive effect of various T lym- itogens on the primary *in vitro* PFC response . Mitogens were added at the time of SRBC and direct anti-SRBC PFC/culture was deter- Day 5. PFC responses are expressed as the luplicate determinations ± SD. The responses sentative of three experiments.

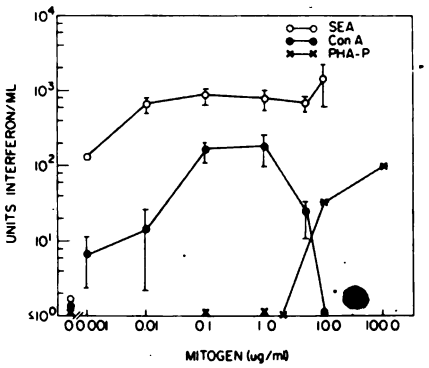


FIG. 2. Stimulation of the production of mitogen-type interferon in C57B1/6 mouse spleen cell cultures by various T lymphocyte mitogens. Spleen cells and mitogens were incubated for 48 hr under conditions as described for the PFC response. Interferon concentrations are expressed as the mean of duplicate determinations ± SD. The SD (not plotted) for 0.001 μg of SEA is 146. The responses are representative of three experiments.

ated, then, for broad correlations at various mitogen concentrations, rather than at a specific mitogen concentration. SEA was the most effective stimulator of interferon; 134 NIH reference units of interferon/ml were produced at 0.001 μg/ml and 644 to 1323 units/ml at 0.01 to 10 μg of SEA/ml. Con A stimulated the cultures to produce 150 and 189 units of interferon/ml at 0.1 and 1.0 μg/ml, respectively. At 0.01 μg of Con A/ml, only 13 units of interferon were produced. At 5 and 10 μg of Con A/ml, the amount of interferon produced significantly declined. This was probably due to the fact that Con A was cytotoxic at these concentrations. The Con A interferon data are consistent with the slight recovery of the PFC response (Fig. 1) at the higher concentrations of Con A. PHA-P was the least effective inducer of interferon; 10 μg/ml stimulated the cultures to produce 32 units of interferon/ml, and 100 μg stimulated 100 units. Lesser concentrations of PHA-P did not stimulate the production of interferon in the cultures. SEA was the most effective inhibitor of the PFC response and was the best inducer of mitogen-type interferon, followed by Con A, with PHA-P being the least effective. This was generally observed both in terms of the concentration of mitogen required for the observed biological activity, and for the extent of the measured activity. Repeated experiments resulted in the same response patterns as illustrated in Figs. 1 and 2, although absolute values varied between experiments.

Kinetic studies (Fig. 3) demonstrated that SEA induction of interferon was substantial at 48 hr of culture, the incubation time used for the data of Fig. 2. A further increase in interferon activity was sometimes observed on Days 3 and 4 of culture. Occasionally a decline in interferon activity was observed on the fifth day of mitogen stimulation of spleen cultures (data not shown). The kinetics of interferon production by SEA-stimulated cultures are similar to those observed for Con A- and PHA-P-stimulated cultures (14). A 48-hr incubation time appears suitable, then, for assessing the relative abilities of these mitogens to stimulate interferon production.

The mitogen-induced interferons are species specific (7, 8). They did not exhibit antiviral activity in human WISH cell lines. It has previously been demonstrated that the mitogen-type interferon is antigenically distinct from virus-type interferon (7, 8); antibodies to virus-type interferon blocked the antiviral activity and PFC inhibitory activity of virus-type interferon, while having no inhibitory effect on mitogen-type interferon.

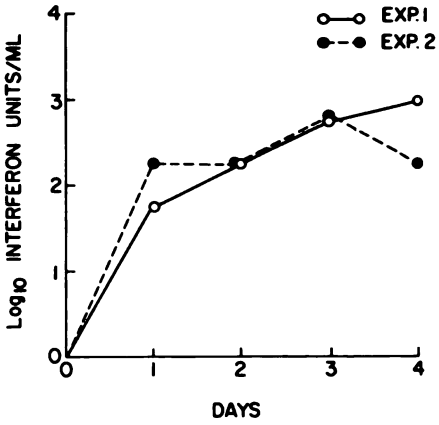


FIG. 3. Kinetics of SEA stimulation of interferon production in C57Bl/6 mouse spleen cell culture. SEA was used at 0.5 μg/ml. The SD (not shown) was similar to those of interferon assays in Fig. 2.

The data are consistent with the interpretation that mitogen-type interferon might be the mediator of mitogen-induced suppressor cell activity. Further, it is possible that mitogen-type interferon, mitogen-induced macrophage migration inhibitory factor (MIF) and mitogen-induced soluble immune response suppressor (SIRS) may be biological expressions of the same substance (7, 8, 15, 16). It has not been possible to separate MIF and antigen-induced interferon (8). Neither has SIRS activity been separated from MIF activity (15). The interferon assay system, as demonstrated here, may be a convenient technique for quantitating these biological activities.

Prerequisite to purification of mitogen-type interferon for characterization and immunization for specific antibodies is a determination of the most suitable inducers. We have shown here that SEA is the most potent inducer of mitogen-type interferon and that this inducer is highly active over a wide

of concentrations. The data presented
re consistent with and supportive of
us studies indicating a relationship be-
mitogen-type interferon and regula-
the immune response by suppressor
(7).

mary. Several T cell mitogens, con-
ılin A (Con A), phytohemagglutinin P
-P), and staphylococcal enterotoxin A
, were compared for their ability to
the *in vitro* antibody response and to
ate the production of mitogen (anti-
type interferon in mouse spleen cell
:s. It was found that the ability to
the plaque-forming cell (PFC) re-
to sheep red blood cells was propor-
to the ability of these mitogens to
interferon in the cultures. SEA was
ost effective inhibitor of the PFC re-
and the best inducer of mitogen-type
ron, followed by Con A, with PHA-P
the least effective. The data suggest
EA would be the most suitable in-
of mitogen-type interferon in quantity
erequisite to purification and charac-
ion of the molecule. The data are sup-
of previous studies suggesting a role
togen (antigen) -type interferon in
ion of the immune response via sup-
r T cells.

ish to thank Ms. Phyllis Myers and Mr. Kley for their skillful technical assistance.

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Received September 7, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 142-145 (1977)

Influence of 2-Acetoamidoethyl (*p*-chloro-phenyl) (*m*-trifluoromethylphenoxy) Acetate (Halofenate) on Hepatic Drug Metabolism in Retired Breeder Rats[1] (39623)

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The relationship between serum cholesterol level and the prevalence of coronary heart disease (1) has stimulated research on plasma lipid-lowering drugs (2). Halofenate is an investigational drug that is closely related structurally to clofibrate (chlorophenoxisobutyrate). This new agent has been shown to lower serum lipids (3, 4) and uric acid (3, 5) in man and plasma cholesterol in rats (6, 7). The long-term safety of halofenate is not known but it has been suggested that it has several advantages over clofibrate; e.g., lower doses are required (8) and it needs to be administered only once daily (4). The chronic administration of clofibrate to rats has produced elevations in liver weight and the proliferation of hepatic smooth-surfaced endoplasmic reticulum (9, 10), and has enhanced rates of hepatic drug metabolism (11). Since halofenate is being considered for use in man as a hypolipidemic agent, and because cholesterol and steroid hydroxylations are presumed to be mediated via the microsomal mixed function oxidase system (12–14), the present study was initiated to ascertain the effect of chronic halofenate administration on several parameters of hepatic drug metabolism in hyperlipidemic retired breeder male rats. The retired breeder rat has been suggested as an animal model for atherosclerosis (15) and hyperlipidemia (16).

Materials and methods. Recently retired Holtzman male breeder rats (500 g) were housed in individual cages and provided tap water and Purina Laboratory Chow *ad libitum* for at least 1 week prior to use. The rats were fasted for 18 hr before collecting tail vein blood for the initial serum lipid determinations. Serum samples were extracted in chloroform–methanol (2:1) and analyzed for cholesterol according to the method of Rudel and Morris (17) and for triglycerides by the method of Eggstein and Kreutz (18), using the Biochemica test combination for triglycerides (Boehringer-Mannheim Corp.). Halofenate (30 or 60 mg/kg) was administered daily for 10 days by stomach tube as a 30-mg/ml solution in corn oil (Mazola), while control rats received corn oil alone. The rats were fasted 18 hr prior to sacrifice.

The rats were killed by decapitation and serum samples were collected. The livers were excised, weighed, and divided into three portions. One part of the liver was homogenized in ice-cold water (1:19 dilution) and analyzed for protein by the method of Lowry *et al.* (19), and another portion of the liver was homogenized in saline (1:8 dilution), extracted in chloroform–methanol (2:1), and analyzed for cholesterol (17) and triglycerides (18). The remainder of the liver was homogenized in 10 vol of 0.25 *M* sucrose and the microsomal fraction was isolated by differential centrifugation as previously described (20). Microsomal protein was estimated by the method of Gornall *et al.* (21), and cytochrome P-450 and cytochrome b_5 were determined by the method of Omura and Sato (22). NADPH–Cytochrome *c* reductase was measured as described by Masters *et al.* (23). Microsomal *N*-demethylation was measured as previously described (20), using ethylmorphine (6.5 m*M*) as substrate. The formaldehyde produced was assayed using the procedure described by Nash (24). Statisti-

[1] Supported by the Medical Research Service of the Veterans Administration, and by NIH Grants No. HL 16363-01 and (NIAMDD) No. P50-AM 18520.

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ıparisons were made using Student's

lts. The results summarized in Table
that halofenate exerted a significant
ıolesterolemic effect on the serum of
breeder rats. The drug administered
dose levels showed a dose-response
Serum triglyceride levels were un-
d after 60 mg/kg of halofenate, and
)f the rats treated with 30 mg/kg of
ıate appeared to increase.
ifferences in body weight were found
n the halofenate-treated and control
breeder rats during the course of this
nent. However, throughout the treat-
eriod 4 of the 11 rats treated with 60
of halofenate died. They were sick
hargic for a day prior to death and
)ward the end of the treatment pe-

ıown in Table II, significant increases
oted in liver weight and liver/body
ratios in the halofenate-treated rats
ı dose levels. Halofenate treatment
affect the levels of hepatic protein or
erol in the retired breeder rats. Liv-
m halofenate-treated animals con-
a significantly higher concentration
yceride than did the controls ($P <$
Microsomal protein was signifi-

cantly increased in the halofenate-treated livers.

An examination of components of the liver microsomal drug-metabolizing system demonstrated that cytochrome P-450 and cytochrome b_5 in both groups of halofenate-treated rats were significantly increased from the control values (Table III). NAPDH-Cytochrome *c* reductase levels were significantly increased only in the rats treated with 60 mg/kg of halofenate. The microsomal rates of ethylmorphine *N*-demethylation were significantly greater in the treated animals in comparison to the control values.

Discussion. The results (Table I) confirm the hypocholesterolemic action of halofenate in rats (6, 7) at dosages (30 and 60 mg/kg) which are 2 and 4 × those usually reported in man (4, 8). However, considerable mortality (4/11) was observed at the higher dosage, which puts the safety of this drug in doubt. Like its parent compound, clofibrate (25), halofenate stimulated hepatomegaly without a concomitant increase in liver protein concentration. In contrast to the findings with clofibrate (25, 26), liver triglyceride concentrations were markedly increased after halofenate treatment, suggesting that the increase in liver weight was

ABLE I. EFFECT OF HALOFENATE ON SERUM LIPID LEVELS OF MALE RETIRED BREEDER RATS.[a]

	Serum cholesterol (mg/100 ml)			Serum triglyceride (mg/100 ml)		
Treatment	Initial	After 10 days of treatment	P[b]	Initial	After 10 days of treatment	P
rol	112 ± 14	114 ± 15	NS	46 ± 8	40 ± 3	NS
fenate (30 mg/kg)	118 ± 17	94 ± 10	<0.02	36 ± 4	49 ± 4	<0.05
fenate (60 mg/kg)	113 ± 12	74 ± 5	<0.05	39 ± 4	38 ± 2	NS

ıes represent the mean ± SE of 7 to 11 rats.
erence between initial and post-treatment determinations.

LE II. EFFECT OF HALOFENATE ON LIVER WEIGHT, LIVER/BODY WEIGHT, AND LIVER COMPOSITION.[a]

		Treatment	
Parameter	Control	Halofenate (30 mg/kg)	Halofenate (60 mg/kg)
ver weight (g)	11.65 ± 0.23	13.71 ± 0.62*	14.35 ± 0.65**
ver/body weight (%)	2.4 ± 0.1	2.8 ± 0.1**	3.1 ± 0.1**
ver protein (mg/g)	204 ± 3	203 ± 3	207 ± 1
ver cholesterol (mg/g)	0.440 ± 0.017	0.476 ± 0.018	0.416 ± 0.022
ver triglycerides (mg/g)	11.9 ± 0.6	26.5 ± 2.1**	23.7 ± 0.92**
icrosomal protein (mg/g)	16.7 ± 1.4	26.1 ± 1.2*	23.8 ± 1.2*

ılts are expressed as the mean ± SE of 7 to 11 rats. Significance: * $P < 0.01$; ** $P < 0.001$.

TABLE III. Measurements of Liver Microsomal Drug Metabolism in Retired Breeder Rats Treated with Halofenate.[a]

	Control	Treatment: Halofenate (30 mg/kg)	Treatment: Halofenate (60 mg/kg)
Cytochrome P-450 (nmole × mg^{-1} protein)	1.11 ± 0.03	1.31 ± 0.06**	1.79 ± 0.05***
Cytochrome b_5 (nmole × mg^{-1} protein)	0.48 ± 0.01	0.52 ± 0.01*	0.59 ± 0.02***
NADPH-Cytochrome *c* reductase (nmole × min^{-1} × mg^{-1} protein)	52 ± 3	62 ± 4	84 ± 4***
Ethylmorphine demethylation (nmole × min^{-1} × mg^{-1} protein)	1.9 ± 0.1	2.8 ± 0.2**	3.4 ± 0.4***

[a] Results are expressed as the mean ± SE of 7 to 11 rats. Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

due, in part, to an increase in lipid content.

Halofenate treatment of rats also increased liver microsomal protein content, stimulated microsomal hydroxylation of ethylmorphine (Table III) and aniline (27), and increased the concentrations of cytochrome P-450 and cytochrome b_5, and increased the activity of NADPH-cytochrome *c* reductase in rat liver microsomes (Table III). These characteristics suggest that halofenate belongs among the more than 200 drugs that are known to stimulate the activity of a variety of hepatic microsomal drug-metabolizing enzymes (28).

These observations suggest that after halofenate treatment in man an increase in the activity of drug-metabolizing enzymes can be expected. This could lead to accelerated biotransformations of drugs and steroid hormones *in vivo* and thus alter the duration and intensity of drug or hormone action.

Only a few reports have attempted to establish the extent of drug-drug interactions associated with the administration of halofenate. Halofenate administration shortened plasma antipyrine and bishydroxycoumarin half-lives but prolonged plasma warfarin half-lives (27) and potentiated the hypoglycemic effect of sulfonylureas (29). Although the mechanism by which halofenate causes these changes is unclear, the present study provides further evidence that this drug, which could be used clinically, both chronically and in combination with other agents, has the potential for altering the metabolism of a coadministered drug.

Summary. Chronic administration of halofenate at two dose levels (30 and 60 mg/kg) to male retired breeder rats caused a significant lowering of serum cholesterol levels. Halofenate treatment also produced hepatomegaly in rats, and this increase in liver weight was due, at least in part, to an increase in the quantity of triglyceride. Considerable mortality (4/11) was associated with the higher dose level. The influence of pretreatment with halofenate on several parameters of hepatic microsomal drug metabolism was investigated. *In vitro* ethylmorphine *N*-demethylation was increased and the concentrations of cytochrome P-450 and cytochrome b_5 were elevated. The specific activity of NADPH-cytochrome *c* reductase was also increased.

The authors thank Merck, Sharp & Dohme Laboratories for a supply of halofenate. We wish to also thank Mrs. Naomi Ross for typing the manuscript.

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Received September 7, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 146-150 (1977)

Effect of Ascorbic Acid and Zinc Sulfate on Ethanol Toxicity and Metabolism[1] (39624)

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Normally, ethanol is metabolized at a constant rate in animals and man. The most successful attempts to accelerate disappearance of ethanol from the blood have been achieved with fructose (1-3) and thyroid hormone (4). However, a number of vitamins (5), amino acids (6, 7), and other nutrients (1) also has been tested with less impressive results.

Krasner *et al.* (8) have shown direct correlations between leukocyte ascorbic acid levels (a good index of total body ascorbic acid stores), the rate of clearance of ethanol from the blood, and the hepatic activity of alcohol dehydrogenase which is responsible for oxidation of ethanol to acetaldehyde and is recognized to be rate limiting in the metabolism of ethanol. However, subsequent work by the same authors has failed to demonstrate any specific *in vitro* effect of ascorbic acid on the activity of alcohol dehydrogenase (9) and no correlation was found between the concentrations of ascorbic acid and alcohol dehydrogenase activity in ascorbic acid-deficient guinea pigs (10).

Zinc is the metal component of alcohol dehydrogenase and plasma concentrations of this metal frequently are low in chronic alcoholics. Prasad *et al.* (11) have shown that hepatic alcohol dehydrogenase activity is decreased to about 60% of normal in zinc-deficient rats when compared to pair-fed control animals. The possibility that supplemental zinc might accelerate ethanol metabolism, perhaps by increasing hepatic activity of alcohol dehydrogenase, has not been tested previously.

Acute and chronic ethanol intoxication has been produced in rats and mice so that the effects of ascorbic acid and zinc supplements alone and in combination on survival and on ethanol metabolism could be examined. Both appear to have protective effects each, alone or in combination.

Methods. The first pilot study was designed to evaluate the chronic protective effects of ascorbic acid on ethanol toxicity in 250-g male Holtzman rats. Mortality rates were observed in four groups (five rats per group) given ip injections five times weekly for 4 weeks. The first group received 56 m*M* ethanol per kg body weight per injection; the second group, 0.56 m*M* ascorbic acid per kg body weight; the third group, an equivalent amount of both ethanol and ascorbic acid; and the fourth group, an equal volume of normal saline which was used as a diluent for both the ethanol and ascorbic acid.

The second series of studies was designed to determine the relative efficacy of ascorbic acid and zinc sulfate alone and in combination on mortality rates in 40-g Cf-1 Charles River female mice with induced ethanol toxicity. Group 2a studies were designed to evaluate the effects of different amounts of the trace metals zinc sulfate and manganese chloride on survival and to rule out any toxicity from the trace elements. Intraperitoneal injections of zinc sulfate (0.2 or 1.0 μM), manganese chloride (0.2 or 1.0 μM), or normal saline (0.5 ml) were given at zero hour. These treatment regimens were followed at 1 hr, 24 hr, and 7 days with ip injections of 4.1 m*M* ethanol. Nine or ten mice were placed in each treatment group. The number of surviving mice was counted on the tenth day.

Larger numbers of mice were studied in Group 2b. Thirty mice received 1 μM Zn^{2+} at zero hour and 35 received saline as a control. One hour later, 4.1 m*M* ethanol was injected ip and the survivors were counted 24 hr later.

In Group 2c, 20 animals were placed in each treatment group. Each of the groups of animals received one of the following alone

[1] Supported by the Medical Research Service of the Veterans Administration.

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mbination: 25 mg of ascorbic acid, , or 1.0 μM zinc as zinc sulfate, or of normal saline. Ethanol injections f) were given at 1 and 24 hr after ı of the above, and the number of g mice was counted after 48 hr.

: third series of studies, 40 450-g oltzman rats were divided into five The first group received 2.5 mM acid per kg body weight in 1.0 ml of he second, 1.4 μM zinc as zinc sul- kg body weight in saline; the third, nation of ascorbic acid and zinc; the .6 mM pyrazole per kg body weight ; and the fifth, 1.0 ml of saline as a

1 hr of equilibration, 15 mM per kg eight of ethanol was injected ip in f saline (40% solution). Blood sam- e drawn at 30, 60, 90, 120, 150, min post-ethanol injection by first the tip of the tail with warm deter- ter, rinsing it with distilled water, he tail under a heat lamp for 5 min, ly slicing off the tip of the tail. The p of blood was discarded and a hep- capillary tube (100 μl) was allowed th blood.

ethanol determinations were per- with the gas chromatographic of Jain (12). A gas–liquid chro- ph (Varian 1200) with Chromosorb , 60/80 mesh column was utilized ethanol analysis, and isobutyl alco- used as the internal standard. Equal (50 μl) of both sample and stan- ntaining known amounts of isobu- ere mixed thoroughly and a 0.5-μl was delivered with a 1-μl Unimetric into the injection port and analyzed ıtely. The nitrogen carrier gas flow ml/min; the oxygen flow, 100 ml/ : hydrogen flow, 30 ml/min; the in- port temperature, 160°; and the nperature, 100°. Following each in- the syringe was rinsed several times : internal standard solution to pre- gging of the needle. The ratios of to isobutanol in unknown and tandards were determined and uti- ı formula to determine ethanol con- ns in mg/100 ml (12). The sensitiv- his method is less than 1 mg/100 ml with a coefficient of variation of ±5%.

Statistical analysis was accomplished with Student's t test for paired and unpaired data.

Results. In the initial pilot study, at the end of 4 weeks, there was 100% survival in the two groups receiving ascorbic acid alone or saline. None of the animals receiving ethanol alone survived. Four of five, however, survived in the group that received ethanol and ascorbic acid in combination.

In the second series of studies in mice, neither zinc sulfate (0.2 or 1.0 μM) nor manganese chloride (0.2 or 1.0 μM) produced any toxicity, as all animals receiving these trace metals survived. The results of the studies in the ethanol-intoxicated animals (4.1 mM) are summarized in Table I. In Groups 2a, only 3 of 10 animals pretreated with ethanol (control) survived the 10 days. Saline (control) survival rate was 100%. Pretreatment with 0.2 or 1.0 μM manganese chloride or 0.2 μM zinc sulfate had no effect on these survival rates. The survival rate in the group pretreated with 1.0 μM zinc was significantly better ($P < 0.05$) than the control group with six of nine animals surviving. In Group 2b, the mice pretreated with zinc sulfate followed in 1 hr by 4.1 mM ethanol showed a 24-hr survival of 77%. This was significantly better ($P < 0.05$) than the control group pretreated with ethanol where only 14% survived.

In Group 2c, only 13 of 40 mice (32%) injected ip with 4.1 mM ethanol on 2 consecutive days survived. In contrast, all 20 mice pretreated with 25 mg of ascorbic acid survived and 18 of 20 (90%) pretreated with zinc sulfate (1.0 μM) survived. Interestingly, smaller amounts of zinc (0.2 and 0.4 μM) improved the survival after the first injection but not after the second.

The third series of studies is illustrated in Fig. 1. Although the zinc- and/or ascorbic acid-treated animals had lower blood ethanol levels 30 min after ip injection of ethanol, these levels did not become significantly less ($P < 0.05$) until 60 min after injection. These differences persisted through the duration of the study. The slopes of the ethanol disappearance curves did not appear to be much steeper in the treated animals. In contrast, the animals

TABLE I. EFFECT OF PRETREATMENT WITH ASCORBIC ACID, ZINC SULFATE (Zn^{2+}), AND/OR MANGANESE CHLORIDE (Mn^{2+}) ON SURVIVAL RATES OF MICE INJECTED ip WITH 4.1 m*M* ETHANOL ON DIFFERENT SCHEDULES (1, 24, AND/OR 168 HR).

11	Number of mice	Treatment 0 hr	Ethanol (4.1 m*M*/ mouse) Times (hr)	Number alive 24 hr	48 hr	10 day	Survival (%)
Group 2a							
1	10	0.2 μM Zn^{2+}	1, 24, 168			3	30
2	9	1.0 μM Zn^{2+}	1, 24, 168			6	67*
3	10	0.2 μM Mn^{2+}	1, 24, 168			4	40
4	10	1.0 μM Mn^{2+}	1, 24, 168			3	30
5	10	Ethanol (control)	1, 24, 168			3	30
6	10	Saline (control)	1, 24, 168			10	100
Group 2b							
7	30	1 μM Zn^{2+}	1	23			77*
8	35	Ethanol (control)	1	5			14
Group 2c							
9	20	Saline (control)		20	20		100
10	20	Ethanol (control)	1, 24	6	6		30
11	20	0.2 μM Zn^{2+}	1, 24	20	4		20
12	20	0.4 μM Zn^{2+}	1, 24	20	2		10
13	20	1.0 μM Zn^{2+}	1, 24	20	18		90*
14	20	Ethanol (control)	1, 24	12	7		35
15	20	25 mg ascorbic acid	1, 24	20	20		100*
16	20	25 mg ascorbic acid + 1.0 μM Zn^{2+}	1, 24	16	16		80*

* Significant difference from controls, $P < 0.05$.

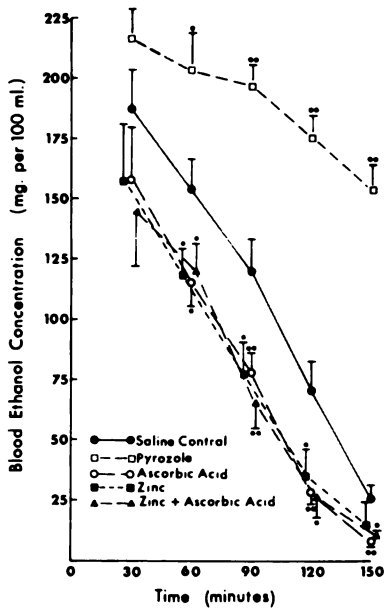

FIG. 1. Blood ethanol concentrations in mg/100 ml of treated groups (pyrazole, ascorbic acid, zinc, zinc and ascorbic acid) and saline control at 30, 60, 90, 120, and 150 min postinjection. Each point represents the mean ± SE of seven rats. Differences from control did not become statistically significant ($P < 0.05$) until 60 min after injection. * $P < 0.05$, ** $P < 0.01$, when treated groups were compared to control.

given pyrazole, an alcohol dehydrogenase inhibitor, had higher ethanol levels 30 min post-ethanol injection and had a much flatter ethanol disappearance curve.

Discussion. The first step in the hepatic elimination of ethanol is the oxidation of ethanol to acetaldehyde. This reaction is catalyzed mainly by alcohol dehydrogenase, an enzyme dependent upon nicotinamide adenine dinucleotide (NAD). Although alcohol dehydrogenase is recognized to be the major enzyme system responsible for oxidation of ethanol to acetaldehyde in the soluble fraction of the liver cell (13), two other systems also have been described, namely an oxidizing enzyme system present in the microsomal fraction of the cell (14) and the NADPH-dependent hydrogen peroxide which metabolizes ethanol to acetaldehyde via catalase (15). Further dehydrogenation of acetaldehyde to free acetate is accomplished by another NAD-dependent enzyme, aldehyde dehydrogenase. The first reaction is the slowest and, therefore, the rate-limiting step, and it appears to be dependent upon the capacity of the liver to reoxidize the NADH formed.

Pawan (1) studied the effects of various

ıins and sugars on the rate of ethanol bolism in man and reported that 600 f ascorbic acid given acutely had no nce on ethanol clearance rates. Kras- *al.* (8) later reported a direct correla- xisted between leukocyte ascorbic acid (an index of ascorbic acid stores), ic alcohol dehydrogenase activity, and learance of ethanol from the blood. ermore, they showed that administra- f 1 g of ascorbic acid daily for 2 weeks l leukocyte ascorbic acid levels and ol clearance rates in 9 of 11 patients. findings of increased survival and de- d blood ethanol levels after a standard ol load in animals pretreated with as- acid partly support their initial obser- ı which, in later publications (9, 10), were unable to confirm.

hough ascorbic acid does not partici- lirectly in the metabolism of ethanol to ldehyde to acetate, it is a strong reduc- ent. The possibility exists that ascorbic an function as an electron donor simi- NAD in ethanol metabolism; hence, it s the NAD/NADH and accelerates the rsion of ethanol to its metabolites.

c is the metal component of the metal- yme, alcohol dehydrogenase. Prasad *et* 1) have shown that hepatic alcohol lrogenase activity is decreased to 60% of normal in zinc-deficient rats compared to pair-fed control animals. ffect of zinc supplementation in nor- nimals has not been reported. Further- no previous studies have been re- l on the effects of zinc supplements on ol clearance or survival rates in ol intoxication. Equimolar quantities nganese chloride failed to provide sim- rotection, suggesting that this is an specific for zinc.

additive or synergistic effect could be ved in providing protection against ol intoxication or in increasing ethanol nce from the blood when the two (ascorbic acid and zinc sulfate) were in combination. Whether these two provide protection against ethanol cation other than that related to more clearance of ethanol from the blood ns undefined.

ıough the blood ethanol concentrations 60 min after injection were significantly lower in the ascrobic acid- and/or zinc sulfate-treated rats, the slopes of the ethanol disappearance curves were not steeper thereafter, suggesting the metabolism of ethanol was not accelerated after 1 hr. Three possible explanations for this observation exist. The first is that ascorbic acid and zinc sulfate delay the absorption of ethanol from the peritoneum. Against this possibility is the failure to see evidence of delayed absorption of ethanol which should tend to flatten the disappearance curve. The second is that ascorbic acid and zinc sulfate increase the volume of distribution of ethanol. This is unlikely as ethanol normally distributes rapidly through all extracellular and intracellular fluid spaces.

The third possibility is that the ascorbic acid and zinc sulfate exert their effects on ethanol metabolism during the first hour, exhausting any effect thereafter. If the increased disappearance of ethanol from the blood were due to increased hepatic alcohol dehydrogenase activity, one would expect to see steeper ethanol disappearance curves in the pretreated animals. The clinical significance of these observations remains to be tested.

Summary. Acute (single dose) and chronic (multiple dose) ethanol intoxication has been produced in rats and mice so that the effects on survival and on ethanol metabolism could be determined in animals pretreated with ascrobic acid and/or zinc sulfate. Only 13 of 40 mice (32%) injected ip with a fixed amount of ethanol on 2 consecutive days survived. In contrast, the survival rates in matched animals pretreated with ascrobic acid (25 mg) or zinc (1 μM) were 100 and 90%, respectively (20 animals per group). Smaller amounts of zinc (0.2 and 0.4 μM) improved the survival after the first injection of ethanol (24 hr), but not after the second (48 hr). Similar observations were made in rats given repeated injections of ethanol over a 4-week period. Serial blood ethanol concentrations were determined in rats pretreated with ascrobic acid, zinc, a combination of ascorbic acid and zinc, pyrazole (an alcohol dehydrogenase inhibitor), or saline (control). Blood ethanol concentrations were significantly

lower ($P < 0.05$) 1 hr after ip ethanol injections in animals pretreated with ascorbic acid and/or zinc when compared to saline control animals. Pyrazole, in contrast, maintained increased blood ethanol levels. These studies indicate that both ascorbic acid and zinc exert protective effects in ethanol-intoxicated rodents.

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Received June 16, 1976. P.S.E.B.M. 1977, Vol. 15

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cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	*M*
degree Celsius (Centigrade)	°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	dia	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	µl	square centimeter	cm^2
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Volume 154, Number 2, February 1977

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Proceedings of The Society for Experimental Biology and Medicine, Vol. 154, No. 2, February 1977. Published monthly except August by Academic Press, Inc., 111 Fifth Avenue, New York, N. Y. 10003. Second class postage paid at New York, N. Y. and at additional mailing offices. Subscription per year $45.00 U.S.A.; $51.50 outside U.S.A. (plus postage). Send notices of change of address to the Office of the Publisher at least 6–8 weeks in advance. Please include both the old and new addresses. Copyright © 1977 by the Society for Experimental Biology and Medicine.

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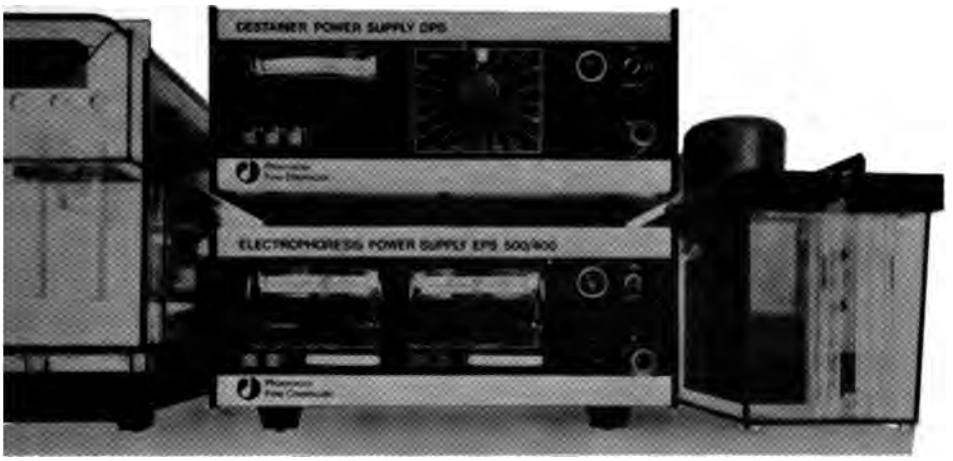

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THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 151-155 (1977)

nd Metabolism of Naphthalene: A Study with Marine Larval Invertebrates (39625)

HERBERT R. SANBORN AND DONALD C. MALINS

anic and Atmospheric Administration, National Marine Fisheries Service, Northwest Fisheries Center, nmental Conservation Division, 2725 Montlake Boulevard East, Seattle, Washington 98112

tion. The toxic effects of water-lrocarbons on marine larvae are of great interest in relation to pe-ontamination of marine waters. udies have examined the acute crude petroleum and water-solu-ns (WSF) on several marine larval 5, 14); however, virtually nothing about the toxicity of individual ons to marine larvae or about the accumulation of petroleum hydro-larval tissues. Moreover, no de-rmation is available on the capa-rly life stages of marine organisms ge accumulated hydrocarbons.

resent work, marine larval inver-ere exposed to 8–12 parts per b) concentrations of [1-^{14}C]naph-r [1-^{14}C]naphthalene complexed ne serum albumin (BSA) in sea-is aromatic hydrocarbon is a component of WSF (6) and is be toxic to marine organisms e findings showed that substantial s occurred in the retention and n of naphthalene between the two forms. In both experiments the e shown to readily depurate naph-lowever, metabolic products to ne were strongly resistant to 1. In each case, 100% mortality hen larvae were exposed for 24-

s and methods. Spot shrimp (*Pan-ceros*) and Dugeness crabs (*Can-r*) were hatched in the laboratory rous females. The shrimp larvae ach day were kept separately in nks and fed brine shrimp. Ani-in the challenge experiments were amorphosed larval stages.

laphthalene (5.10 mCi/mmole) ed from Amersham-Searle, Ar-leights, Ill. Solutions of [1-^{14}C]naphthalene (A), [1-^{14}C]naphthalene-BSA (B), and BSA free of naphthalene (C) were prepared. [1-^{14}C]Naphthalene (1.8×10^7 dpm) was added to a 3-liter portion of a 6-liter solution of salt water buffered with phosphate (pH = 7.5) to form solution A. Solution B was obtained by adding 1.50 g of BSA and [1-^{14}C]naphthalene (1.8×10^7 dpm) to the remaining 3 liter. Then, the naphthalene-BSA complex was formed according to the procedures of Sahyun (10). Solution C was treated in a manner similar to solution B, excluding the [1-^{14}C]naphthalene to provide water for a control experiment.

Solutions were obtained for challenge experiments by mixing the above refrigerated (1°C) stock solutions (A, B, and C) with filtered sea water (5 μm). Nine-liter test aquaria were filled by simultaneously adding 7.80 liter of sea water and 1.65 liter of the appropriate stock solution. A peristaltic pump maintained constant flow rates of 1.1 ml/min for the stock solutions and 5.2 ml/min for the sea water during the exposure period. The resulting solutions were delivered to test aquaria which were maintained at 10±1°. Duplicate water samples were taken at the initiation of the experiments and concurrently with larval samples. These were analyzed for carbon-14 by liquid scintillation spectrometry. The concentrations of [1-^{14}C]naphthalene in aquaria waters ranged from 8–12 ppb on the basis of measured carbon-14 (^{14}C) and the specific activity of [1-^{14}C]naphthalene. The larvae were netted from the holding tanks, counted, and placed in the test aquaria. Specimens were removed for analysis at various time intervals.

Larval tissues were examined for both [1-^{14}C]naphthalene and ^{14}C-labeled metabolites. The total ^{14}C in the animals was determined as follows. Two larvae were sampled

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from each challenge experiment; each was placed in a cone of Whatman 42 filter paper and was washed 10 times with 10-ml aliquots of filtered (5 μm) seawater. The final washings were shown to be free of measurable ^{14}C. Larvae were weighed, and each animal was placed in a scintillation vial with 1.0 ml of soluene 350 (Packard Inst. Co., Downers Grove, Ill.). The tissues were digested for 1 hr at 25°, then, 15 ml of InstaGel (Packard Inst. Co.) was added, and ^{14}C was determined by liquid scintillation spectrometry. The total metabolites of [1-^{14}C]naphthalene were determined by employing methods described previously (11). Two larvae were washed as above, frozen, and held at −10°. Each frozen larvae was crushed in a 5-ml glass-stoppered graduated centrifuge tube containing 0.7 ml of formic acid and 1.5 ml of hexane. The tissues were held at room temperature in stoppered tubes until digestion was complete (12–24 hr). Upon completion of digestion, 1.0 ml of distilled water was added. Then 1.0 ml of a saturated aqueous solution of sodium hydroxide was added slowly with rapid stirring at 0°. An additional 0.5 ml of hexane was added, and the resultant mixture was agitated vigorously and allowed to stand for 5 min. The ^{14}C in the aqueous phase was determined by analysis of a 1.0-ml aliquot using liquid scintillation spectrometry. Background ^{14}C levels were measured by following the above procedure exclusive of animal tissue. The amount of [1-^{14}C]naphthalene was obtained by subtracting the ^{14}C associated with the aqueous phase of the formic acid digest from the total ^{14}C in the larvae. Analysis of test aquaria water by the above formic acid procedure revealed that virtually all of the ^{14}C (determined by liquid scintillation spectrometry) was in hexane washings. Hence, radioactivity was in the form of naphthalene rather than as products of biodegradation.

The naphthalene uptake and depuration data were treated statistically by fitting regression equations to the uptake data for the naphthalene and BSA-naphthalene exposures. Regression equations were also fitted to the depuration data for both exposures. Correlation coefficients for the naphthalene and BSA-naphthalene uptakes were 0.650 and 0.509, respectively, and, for depuration, the coefficients were 0.740 and 0.620, respectively. A statistical treatment of the data indicated that the slopes of the regression equations for naphthalene and the protein complex were the same during uptake as during depuration. However, by analysis of covariance, it was shown that the regression does not coincide during either uptake or depuration: $F = 2.374$ ($F_{05[1,38]} = 4.08$) and $F = 0.699$ ($F_{05[1,66]} = 4.00$). Graphs of the regression equations are shown in Figs. 1A and 2A. In the graphs of metabolite data (Figs. 1B and 2B) curves of mean values and ranges are given.

Results and discussion. [1-^{14}C]Naphthalene and the [1-^{14}C]naphthalene-BSA complex were acutely toxic to stage I and V spot shrimp (*P. platyceros*) and to newly hatched Dungeness crab (*C. magister*) zoea at naphthalene concentrations of 8–12 ppb ($8–12 \times 10^{-9}$ mg/ml). All organisms died within 24–36 hr. In each case, narcosis was observed in 18–24 hr. No differences

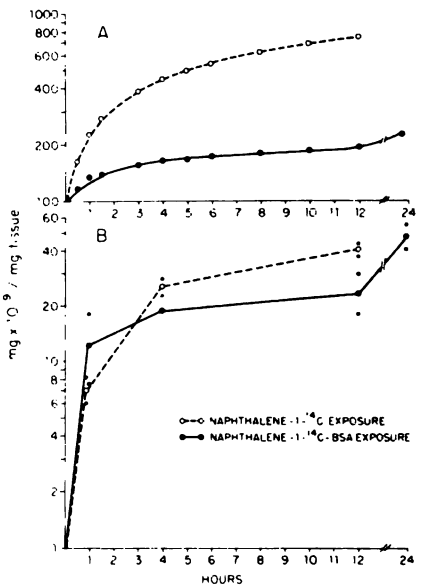
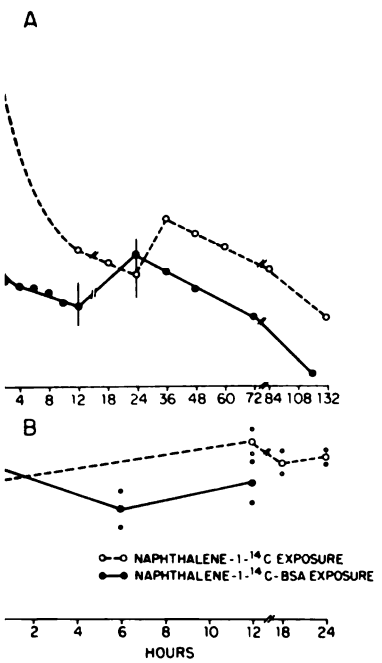


FIG. 1. Accumulation of naphthalene and metabolic products (expressed as naphthol) on exposure of stage V spot shrimp (*Pandalus platyceros*) to 8–12 ppb of water-borne [1-^{14}C]naphthalene and [1-^{14}C]naphthalene complexed with BSA. (A) Regression lines of concentrations of [1-^{14}C]naphthalene with sampling points indicated. (B) Median values of metabolic products with data ranges.



)epuration of naphthalene and metabolic
‹pressed as naphthol) after exposure of
shrimp (*Pandalus platyceros*) to 8–12 ppb
ne [1-^{14}C]naphthalene (12 hr) and [1-^{14}C]-
complexed to BSA (24 hr). (A) Regres-
concentrations of [1-^{14}C]naphthalene with
ints indicated by vertical lines; thereafter,
:tabolic products containing small amounts
phthalene. (B) Median values of metabolic
h data ranges.

:d between larvae exposed to [1-
halene or the [1-^{14}C]naphthalene
omplex. Less than 1% mortality
in the control groups, thus indicat-
SA did not contribute to the toxic
served.

;ults are not consistent with find-
by McAuliffe *et al.* (12), suggest-
arval stages of marine organisms
resistant to petroleum hydrocar-
15). Under our conditions, naph-
ppeared to be considerably more
nvertebrate larvae than WSF of
troleum administered via static
(16). These workers found that
s did not occur among stage I
;s crab (*C. magister*) and tanner
onoecetes bairdi) in 48-hr and 96-
res to 8 ppm of the WSF of crude

petroleum. This level corresponds to a naphthalene concentration approximately five times greater than concentrations of naphthalene used in the present work (17).

It is not possible to compare directly our findings with those obtained using the static bioassay technique, notably because low molecular weight hydrocarbons, such as naphthalene, are volatilized under static conditions. Static bioassay techniques, thus, are largely restricted to providing a rough first-order approximation of the toxicity of water-soluble hydrocarbon fractions to marine larval forms in view of the present data. In considering this question, it is interesting to note that laboratory preparations of WSF of four crude oils were shown to contain from 20–840 ppb of naphthalene (6). This range is well in excess of the acutely toxic concentration of 8–12 ppb found in the present work.

Stage V spot shrimp larvae (*P. platyceros*) were studied with respect to the accumulation of [1-^{14}C]naphthalene and metabolic products. Also studied was the capacity of larvae to depurate these compounds when placed in clean water. The larvae showed significant differences in accumulations of naphthalene when it was presented in the free state rather than as the naphthalene-BSA complex (Fig. 1A). Maximum levels of 820 ppb (818×10^{-9} mg/mg of tissue, 10.0 hr) and 220 ppb (221×10^{-9} mg/mg of tissue, 5.0 hr) were found in larvae exposed to naphthalene and naphthalene-BSA, respectively. These values represent an accumulation of naphthalene in larvae equal to 25–100 times the concentration in the water.

The maximum concentration of naphthalene metabolites (based on the molecular weight of naphthol) in larvae after 12 hr of naphthalene exposure was 42 ppb (41.8×10^{-9} mg/mg of tissue); larvae exposed to naphthalene-BSA for 24 hr contained (based on the molecular weight of naphthol) 49 ppb (49.2×10^{-9} mg/mg of tissue) (Fig. 1B). The former and latter values represent 9 and 21% of the total radioactive compounds isolated from the larval tissues. At each interval of time, the ratio of naphthalene metabolites:naphthalene was substantially greater with larvae exposed to naph-

thalene-BSA than with larvae exposed to naphthalene alone. Thus, a substantially greater proportion of metabolic products occurs in larvae exposed to naphthalene complexed with protein. Although this finding is not readily explained, the data clearly lead to the conclusion that naphthalene metabolism is altered in relation to whether the hydrocarbon is in the free state or bound to protein. The fact that the larvae are capable of metabolizing naphthalene in either chemical form suggests the presence of degradative enzyme systems (aryl hydrocarbon hydroxylases) common to most animals (18). Increased lipid solubility has been shown to be directly related to adsorption through the intestinal mucosa in rats (19); thus, increased uptake of naphthalene over the naphthalene-BSA complex may be attributed to the higher lipid solubility of the former structure. The apparent increase in the proportion of metabolic products found with the naphthalene-BSA exposure cannot be explained at this time.

The question arose as to whether the accumulated levels of hydrocarbons and metabolites are readily depurated. It was shown that transfer of the larvae to clean salt water after exposure to [1-^{14}C]naphthalene or [1-^{14}C]naphthalene-BSA for 12 and 24 hr, respectively, resulted in substantial depuration. Moreover, mortalities that normally occurred on continued exposure for 24–36 hr were prevented. In naphthalene-exposed larvae, a rapid decline in the aromatic hydrocarbon levels over a 12-hr period was observed, followed by a gradual decrease for 132 hr (Fig. 2A). After 24 hr, the concentration of naphthalene decreased by 83% of the original values. A decline of 81% in accumulated naphthalene occurred in larvae exposed to naphthalene-BSA in a 12-hr period (Fig. 2A). Thus, regardless of the chemical form of naphthalene, substantial amounts of the hydrocarbon were depurated from larvae after termination of the exposure. However, metabolites were present in the larvae throughout the depuration experiments (Fig. 2B).

The present study provides details, for the first time, on the toxic and biochemical effects of water-borne naphthalene on larval invertebrates. The results, showing that death (preceded by narcosis) occurred from exposure to low parts per billion levels of naphthalene in 1 or 2 days, raises questions about the nature of long-term effects (e.g., alterations in cellular structure, molting behavior, etc.) at even lower hydrocarbon concentrations.

The finding that the proportion of metabolites to recovered aromatic hydrocarbon is greater with larvae exposed to the naphthalene-BSA complex than with those exposed to naphthalene itself is of interest with respect to the transport of aromatic hydrocarbons in ecosystems. That is, aromatic hydrocarbons acquired in food may be metabolized quite differently than such compounds acquired via other routes (e.g., through the water column), involving other chemical forms.

Summary. [1-^{14}C]Naphthalene and the [1-^{14}C]naphthalene complexed with BSA at 8–12 ppb in flowing sea water produced 100% mortality in 24–36 hr in newly hatched Dungeness crab (*Cancer magister*) zoea and stage I and V spot shrimp (*Pandalus platyceros*). Maximum accumulation of [1-^{14}C]naphthalene in the stage V spot shrimp exposed to [1-^{14}C]naphthalene was nearly four times greater than in shrimp exposed to the [1-^{14}C]naphthalene-BSA complex (820 and 220 ppb, respectively). These values represent a magnification of 25–100 times the exposure levels. Metabolic products of [1-^{14}C]naphthalene in larval tissues exposed to the complexed and noncomplexed naphthalene reached a maximum of 21 and 9%, respectively, of the total radioactive compounds (based on the molecular weight of naphthol). [1-^{14}C]Naphthalene was almost entirely depurated from the tissues in 24–36 hr, whereas metabolic products were strongly resistant to depuration.

This research was supported in part by NOAA's Environmental Research Laboratories Outer Continental Shelf Environmental Assessment Programs under funding from the Bureau of Land Management.

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Received April 26, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 156-159 (1977)

Functional Maturation of Mouse Thymus Cells (39626)

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Details of the functional development of each of the various lymphoid cells involved in immunological reactions is needed for understanding the events controlling the onset of immunological competence in an animal. It is only recently that immunologists have concentrated their efforts at establishing the ontogenic pattern of emergence of structural and functional parameters of immunocompetent lymphoid cell populations (1-7).

Regarding mouse thymus cell maturation, it has been shown that the maximal responsiveness of thymus cells to different mitogens arises at different stages of ontogeny (2, 8-10). Thymus-specific antigens can be detected on thymus cells beginning at Day 16 of development (11), i.e., as soon as there are detectable thymic small lymphocytes; on the other hand the ability of thymus cells to cause a graft-versus-host reaction arises several days later, around the time of birth (12, 13). Using an *in vivo* reconstitution technique, Chiscon and Golub (14) demonstrated thymus helper cell function in the immune response to sheep erythrocytes 24 hr prior to birth whereas Claman *et al.* (15) found such activity only 6 days after birth.

The present study was designed to determine the onset of the capacity of murine thymus cells to show helper function in the response to sheep erythrocytes (SRBC) using entirely *in vitro* methods. Thymus cells directly from thymus usually cannot provide the helper function *in vitro* unless activated with antigen in irradiated host (16) or mitogen *in vitro* (17). Treatment with mitogenic lectin concanavalin A (con A) was employed for our purpose. The study demonstrates that, as early as the sixteenth day of embryonic development, the thymus contains cells that can readily be activated *in vitro* to function as helper cells in the humoral immune response. The effect of con A on the expression of thymus cell surface antigens has also been reported in this paper.

Materials and methods. BDF_1 adult male mice and BDF_1 embryos (C57Bl/6 × DBA/2) from time pregnancies from our colony have been used in this study. Thymus cells, 3×10^6 per petri dish (No. 3001, Falcon Plastic, Calif.), were pretreated with 5 μg of concanavalin A per ml (3X crystallized; Miles Laboratories, Inc., Elkhart, Ind.) for 24 hr at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Minimum Essential Medium (Microbiological Associates, Bethesda, Md.), which had been supplemented with 10% heat-inactivated fetal bovine serum (Colorado Serum Co., Denver), glutamine, and antibiotics, was used for thymus cell culture. After two washes, 3×10^6 con A-treated or -nontreated thymus cells were combined with 5×10^6 bone marrow-derived B spleen cells. The B spleen cells were obtained from adult thymectomized mice X-irradiated with 850 R and reconstituted with syngeneic bone marrow. The combined cell suspension was cultured with about 1.2×10^7 SRBC (GIBCO, Madison, Wis.) as antigen for 4 days as described by Click and coworkers (18). Antibody-forming cells were assayed according to the method of Cunningham and Szenberg (19).

The relative difference in the expression of θ and H-2 antigens was measured by determining the amount of radioactivity released from Cr^{51}-labeled thymocytes exposed to anti-θ or anti-H-2 sera in the presence of complement; the dilution of anti-θ and anti-H-2 sera were such that the sera killed about 50% of adult thymus and bone marrow cells, respectively. The anti-θ serum was raised in AKR mice by repeated injections of thymus cells from C3H mice. To raise the anti-H-2 serum, CBA ($H\text{-}2^k$) mice were repeatedly injected with BDF_1 (H-

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ISSN 0037-9727

[b,d]) spleen cells. The percentage of cytotoxicity was calculated as:

$$\frac{\text{Mean counts per minute of experimental release} - \text{Mean counts per minute of spontaneous release}}{\text{Mean counts per minute of maximum release} - \text{Mean counts per minute of spontaneous release}} \times 100.$$

Spontaneous release was defined as the level of Cr^{51} released in the presence of normal mouse serum and complement. Maximum release was obtained by rapidly freeze-thawing the labeled cells three times.

Results. The pattern of *in vitro* collaboration between B spleen cells and con A-treated and -nontreated thymus cells from adult and newborn mice is presented in Fig. 1. Only con A-treated thymus cells provided the helper function *in vitro;* other cell types, such as bone marrow (BM) cells, could not provide this function even after treated with con A. Using this *in vitro* technique of collaboration, it has been shown that mouse thymus cells, as early as the sixteenth day of embryonic life, develop the functional potential to collaborate with B spleen cells in mounting an antibody response against SRBC (Table 1). However, this potentiality of thymocytes could only be revealed by triggering them with con A. With an increase in age, the collaborating ability of embryonic thymus cells increases. The re-

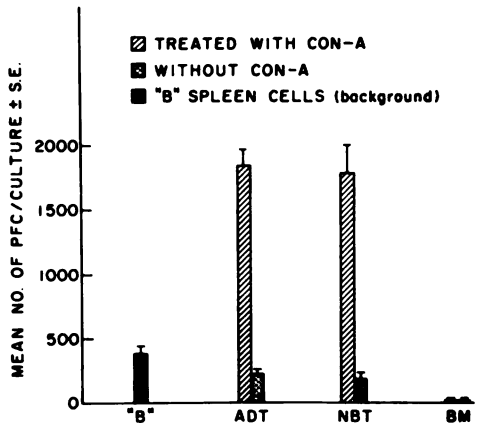

FIG. 1. *In vitro* collaboration between bone marrow-derived spleen (B) cells and con A-treated adult (ADT) or newborn (NBT) thymus cells, as measured by the number of plaque-forming cells (PFC) per culture. Each culture contains 5×10^6 B cells and 3×10^6 thymus or bone marrow (BM) cells with or without con A pretreatment for 24 hr, and each culture is immunized with about 1.2×10^7 SRBC. The PFC response of 5×10^6 B cells is the background response. Five experiments were performed with adult and newborn thymus cells and three experiments with bone marrow cells, each experiment consisting of five replicate cultures in each category; B-cell controls were maintained with each experiment.

TABLE I. ABILITY OF THYMOCYTES FROM DIFFERENT STAGES OF ONTOGENY TO COLLABORATE WITH ADULT B SPLEEN CELLS *in Vitro*.[a]

Types of T cells added to B spleen cells	Mean PFC/culture ± SE (Number of culture)		P[b]
	Without Con A	With Con A	
Adult	612 ± 52 (15)	2735 ± 446 (14)	<0.01
Newborn	520 ± 133 (8)	1644 ± 354[c] (10)	<0.05
18-Day embryonic	96 ± 32 (13)	2466 ± 466 (15)	<0.01
17-Day embryonic	117 ± 34 (20)	1726 ± 212 (19)	<0.01
16-Day embryonic	170 ± 54 (8)	1087 ± 137 (14)	<0.01
15-Day embryonic	167 ± 88 (5)	584 ± 300 (5)	>0.01
B spleen cells (background)	703 ± 72 (43)		

[a] T Cells, 3×10^6, treated with or without con A for 24 hr, combined with 5×10^6 B spleen cells, and immunized with 1.2×10^7 SRBC; assayed on fourth day.

[b] Statistical significance of the difference between the PFC response obtained after addition of con A-treated thymus cells and B spleen cells (background response).

[c] The difference between the response of 18-day embryonic and adult thymocytes is not statistically significant.

sponse of 18-day-old embryonic thymus cells was almost identical with that of adult thymus cells.

After con A treatment of thymus cells, the percentage of cytotoxicity with anti-θ serum decreased and a corresponding increase in the percentage of cytotoxicity with anti-H-2 serum was observed; in contrast to its effect on thymus cells, con A caused no detectable change in surface antigens of bone marrow cells (Fig. 2).

Discussion. The present investigation demonstrates that thymocytes obtained from mouse embryos can exert demonstrable helper cell activity in an *in vitro* antibody response when they are activated by 24 hr of incubation with con A; indeed, as soon as small lymphocytes appear in the thymus, around the sixteenth day of gestation, they appear to have the potential for this immunological activity. The collaborative ability of embryonic thymocytes increased rapidly, reaching adult levels before birth. This increase appears correlated with the relative proportion of small lymphocytes found in the developing thymus (20) which also correlates with the relative efficiency with which con A and phytohemagglutinin induce thymidine incorporation by embryonic thymus cells (10).

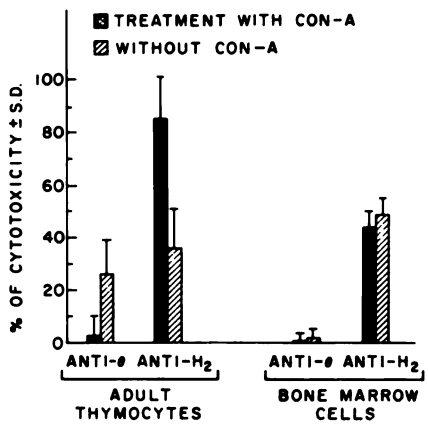

FIG. 2. Effect of con A on θ and H-2 antigen expression on adult thymocytes and bone marrow cells, as measured in terms of the susceptibility of con A-treated and -nontreated cells to the specific antiserum. This figure is representative of one experiment consisting of triplicate values per point. Similar results were obtained in four other experiments using the same type of antisera and in three experiments using antisera raised in congenic mice (latter experiments will be reported somewhere else).

There may be several reasons for our ability to demonstrate immunocompetence of thymus cells during embryonic life. An *in vitro* test system, like ours, in contrast to the studies cited above (14, 15), does not encounter some of the difficulties of the *in vivo* reconstitution technique, such as the problem of the seeding efficiency of injected cells or the possibility of the functional potency of the cells being masked *in vivo* by one or more host factors.

In the present investigation, thymus was used instead of spleen as the source of thymocytes. Thus, our results are not in contrast with those of Spear and Edelman (5) who have shown that con A-responsive T cells and T-cell helper function could not be detected in the spleen cells from 1-day-old mice.

That the nature of con A activation is meaningful in terms of the development of immunocompetence of thymus cells is suggested by several ancillary findings. (i) Con A bound to bone marrow cells rather than to thymus cells cannot substitute for thymus cells in an antibody response (see Fig. 1). (ii) Con A bound to thymus cells does not immediately activate those cells; rather the activation occurs over a 24-hr period (17) (unpublished observation). (iii) It is considered that con A-mediated stimulation of T lymphocytes closely resembles lymphocyte activation by specific antigen in morphologic as well as in certain functional aspects (21). (iv) The shift in the percentage of cytotoxicity with a specific antiserum (see Fig. 2) may reflect on the different degree of expression of the specific antigen on the con A-treated T-cell surface. The possibility of the selection of T cells with a lower amount of θ and a higher amount of H-2 by cell death seems unlikely. Even if con A were to select the variety of cortisone resistant functional thymocytes having a lower amount of θ and a higher amount of H-2, these normally constitute only about 5% of the total thymocytes in a thymus, and, hence, do not account for 30% to more than 60% recovery of thymus cells in different experiments after 24 hr of con A treatment (unpublished observation). It is then possible that incuba-

tion with con A caused a decrease in the expression of θ antigen and a corresponding increase in the expression of H-2 antigen. This type of shift in surface antigens (high to low θ; low to high H-2) is known to occur during the final steps of thymus cell maturation (22). (v) As soon as the embryonic thymus cells can be activated for helper function *in vitro* (see Table 1), the con A-induced shift in the expression of these cell surface antigens can also be detected (to be published).

As helper function of thymus cells treated with con A is the main concern of this study, we are not going into a detailed discussion of con A-induced inhibitory T cells.

The early detection of mouse thymus cell helper function in an antibody response tends to argue against a requirement for an extended period in ontogeny during which a somatic process of generation of immunological diversity takes place. On the other hand, the results presented in this paper possibly provide some support for the discovery that antigen-binding cells can be detected early in the development of mouse thymus (3). However, as there are evidences of cross-specificities of T-cel humoral factor, only further experimentation showing the antigen specificity of con A-treated helper T cells will provide clarity in the argument against somatic process of generation of immunological diversity.

Taken together with the finding of early differentiation of cells from the embryonic liver and yolk sac to perform various functions associated with cell mediated immunity (23, 24), it seems clear that we can no longer think of young mammalian embryos as immunologically inert.

Summary. Ontogeny of thymus cell function in humoral antibody response was studied *in vitro* using cells from different embryonic stages and neonates of BDF_1 mice. As early as the sixteenth day of embryonic development, the thymus contained cells that were readily activated *in vitro* by con A to function as helper cells in an antibody response against sheep erythrocytes. The helper function of embryonic thymocytes increased rapidly, reaching adult levels before birth. It has also been shown that con A-treated thymus cells are less susceptible to anti-θ serum and correspondingly more susceptible to anti-H-2 serum; the implication of this finding in thymus cell maturation has been discussed.

The author is grateful to Professor Robert Auerbach for his encouragement and criticisms during the course of this work. This study was supported in part by Research Grant CA 13548 from the National Institute of Health.

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Received May 19, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 160-163 (1977)

Toxicity of 5-Bromo 2'-Deoxyuridine to Malignant Lymphoid Cell Lines (39627)[1]

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The thymidine analog, 5-bromo 2'-deoxyuridine (BUDR) has diverse biological properties including radiosensitization (1-3), induction of latent viruses (4, 5), inhibition of expression of both tissue-specific differentiated traits (6, 7) and oncogenic potential of malignant cells (8, 9). BUDR is acutely cytotoxic to many cell cultures at concentrations above 10 μg/ml (10, 11). The data reported here show that various malignant lymphoid cell lines are sensitive to the toxic effects of BUDR at concentrations which are not cytotoxic to other cell types. A similar report describing the toxicity of BUDR to one line of mouse myeloma has recently appeared (12).

Material and Methods. *Cell lines.* MOPC 315 and MPC 11 are murine myelomas given to us by Dr. Herman Eisen and Dr. Matthew Scharf, respectively. S49.1 is a θ-positive murine lymphoma. S49.1TB.2 is a clone of S49.1 that can be grown in 30 μg of BUDR/ml. Both S49.1 and S49.1TB.2 were given to us by the Salk Instiute, La Jolla, Calif. RAG is a clone from a murine renal adenocarcinoma. CCRF/CEM is a lymphocytic cell line derived from the peripheral blood of a 4-year-old female with acute lymphoblastic leukemia. MMT is a murine mammary carcinoma. RAG, CCRF/CEM, and MMT were from the American Type Culture Collection, Bethesda, Md. L929 is a clone of mouse fibroblasts, and HeLa is derived from a human cervical adenocarcinoma. Both L929 and HeLa were purchased from Microbiological Associates, Bethesda, Md.

Cell cultures. All cultures were routinely carried in 250 ml (75 cm^2) Falcon tissue culture flasks containing 10 ml of media under a gas phase of 5% CO_2-95% air. They were fed 2 or 3 times weekly. Monolayer cultures were trypsinized with 0.25% trypsin and diluted into fresh flasks approximately once each week.

MOPC 315, MPC 11, L929, S49.1, and S49.1TB.2 were carried as suspensions in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% heat-inactivated horse serum kindly provided to us by the New York City Public Health Department. RAG cells were carried in DMEM with 10% fetal calf serum (Microbiological Associates, Bethesda, Md.). HeLa cells were grown in Minimal Eagle's Medium (MEM) containing 10% pooled human serum, or, when grown in suspension, in Ca^{++} and Mg^{++}-free MEM with 10% human serum. MEM and DMEM (both from Grand Island Biological Co., Grand Island, N.Y.) were fortified with nonessential amino acids. All media contained 100 units of penicillin, 100 μg of streptomycin, and 100 μg of gentamycin per ml.

Experimental procedure. Experiments were carried out in 2 ml cultures using 24 well Linbro plates. BUDR solutions of 1 mg/ml were made up weekly in Hanks' balanced salt solution and diluted into the appropriate media. All manipulations of BUDR or BUDR-containing solutions were done under Kodak 1A safelights; cultures of media containing BUDR were kept strictly in the dark.

Proliferation of cell cultures was assayed by determining the number of Trypan blue-excluding cells on the days indicated. All P values of ≤ 0.05 by the Student's t test are shown.

Nucleosides. BUDR and thymidine were purchased from Sigma Chemical Co., St. Louis, Mo. The nucleosides were shown to be pure when chromatographed on Silica thin-layer plates (Brinkmann Instruments, Long Island, N. Y.) using the following solvent systems: *n*-butanol (50%), acetic acid (25%), and water (25%); isopropanol

[1] This investigation was supported by Public Health Service Research Grants Nos. CA 10064 and CA 14194 from the National Cancer Institute.

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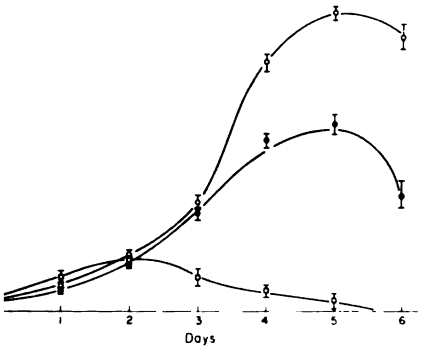
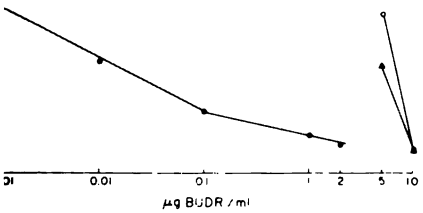
ISSN 0037-9727

, water (20%), and 10^{-3} *M* EDTA; stilled water alone.

ılts. *Effect of BUDR on the prolifera-*
of malignant lymphoid cell lines.
h of various malignant lymphoid cell
ı the presence of 0.10 or 1.0 μg of
l/ml results in marked toxicity, appar-
the third day of growth as shown in
. and 2 for mouse myeloma MOPC
ells seeded in prelog phase undergo
imately five doublings before reach-
saturation density of 1.5–2.0 × 10^6
il. In the presence of 1 μg of BUDR/
growth rate is similar to that of the
ls for approximately the first 24–48 hr
ıre, after which there is a progressive
ı both total and viable cell counts. In
l-treated cultures, the percentage of



1. Effect of 1.0 and 0.1 μg of BUDR/ml on
/th of MOPC 315. Triplicate 2 ml cultures were
ıt an initial cell density of 8 × 10^4 cells/ml. One
as counted each day and discarded. Culture
ns were as described in Materials and Methods.
.0 (□), and 0.1 (●) μg of BUDR/ml.



2. Times necessary for the toxic effect of
to MOPC 315 (●), HeLa (▲), and L929 (○).
re mean values calculated from several growth
ents. Toxicity is defined as statistically signifi-
ıibition of cell proliferation as compared to
ıultures.

live cells drops from over 90% on Day 1 to less than 20% on Day 5.

The growth curves of mouse myeloma MPC 11, mouse lymphoma S49.1, and human acute lymphoblastic leukemia CCRF/CEM follow qualitatively similar kinetics in the presence of BUDR.

Table I (Group I) shows the concentration of BUDR needed to cause a 50% inhibition of cell proliferation after 4 days of exposure to the analog. The doses range from 0.09 to 0.31 μg of BUDR/ml for the malignant lymphoid cell lines studied except for S49.1TB.2, which requires 87.0 μg of BUDR/ml.

Concentrations of 0.01 and 0.001 μg of BUDR/ml are toxic to MOPC 315 after repeated subcultures in its presence. As shown in Fig. 2, there is an inverse relationship between the time necessary for the expression of the toxic effect of BUDR and the amount of analog present.

Growth of other cell lines in the presence of BUDR. A variety of other cell types can be grown in BUDR concentrations that are extremely toxic to malignant lymphoid cell lines. Table I (Group II) shows that concentrations of BUDR ranging from 3.14 to 6.23 μg/ml are required for a 50% inhibition of proliferation. Concentrations of BUDR between 1 and 2 μg of BUDR/ml occasionally cause a transient inhibition of proliferation which is generally followed by a stimulation of growth compared to nontreated cultures.

TABLE I. CONCENTRATION OF BUDR REQUIRED TO CAUSE A 50% INHIBITION OF CELL PROLIFERATION IN 4 DAYS (ID_{50}).[a]

Group	Cell line	ID_{50} (μg/ml)
I	MOPC 315	0.09
	MPC 11	0.20
	CCRF/CEM	0.31
	S49.1	0.16
	S49.1TB.2	87.0
II	RAG	3.70
	L929	6.23
	HeLa (monolayer)	3.14
	HeLa (suspensions)	4.20
	MMT	4.25

[a] The 50% inhibitory doses (ID_{50}) of BUDR were determined from a graph of inhibition vs dose for 4 days of growth in the presence of the analog.

TABLE II. PROTECTION BY THYMIDINE AGAINST THE T LYMPHOID CELL

Condition	MO
Control	1
1 μg of BUDR/ml	
(1 μg of BUDR + 1 μg of thymidine)/ml	
1 μg of Thymidine/ml	
(1 μg of BUDR + 10 μg of thymidine)/ml	
10 μg of Thymidine/ml	

[a] Triplicate 2 ml cultures were seeded at an initial cell
Material and Methods. Data are given as the percentage
[b] $P \leq 0.001$.
[c] $P \leq 0.005$.
[d] $P \leq 0.01$.
[e] $P \leq 0.025$.

Effect of thymidine on the toxic effect of BUDR to malignant lymphoid cells. As shown in Table II, the toxicity of 1 μg, of BUDR/ml to mouse myeloma cells is completely reversed by 10 μg of thymidine/ml. The effect on mouse lymphoma S49.1 is fully prevented by 1 μg of thymidine/ml. Thymidine alone either has no effect or slightly stimulates the proliferation of these cultures.

Discussion. Malignant lymphoid cell cultures are sensitive to the cytotoxic effects of BUDR at concentrations which are not harmful to other cell lines. This toxicity is dependent on both the concentration and duration of exposure to the analog.

Although the spectrum of acute toxicity at low levels of BUDR extends to two mouse myelomas, a mouse T-cell lymphoma, and a human acute lymphoblastic leukemia, reports of the continued high viability of human Burkitt lymphoma cells exposed to BUDR (13) suggest that the pronounced cytotoxicity observed here does not extend to all malignant lymphoid cell cultures.

The toxicity of BUDR at the concentrations used in this study is specific for malignant lymphoid cells of both T and B-cell origin. It is not dependent on the media and serum used or whether the cells grow as adherent or suspension cultures.

BUDR competitively replaces thymidine in DNA that is synthesized in its presence (11, 14). In most systems, the effects of BUDR are prevented by either blocking DNA synthesis (15) or by the addition of excess thymidine (12, 16–18). Our results indicate that the incorporation of BUDR into the DNA of malignant lymphoid cells

occur with mouse lymphoma S49.1, leukemia CCRF/CEM, and mouse a MPC 11. The dose of BUDR re- o cause a 50% inhibition of proliferD$_{50}$) ranges from 0.09 to 0.3 μg of 'ml. In contrast, the ID$_{50}$ for a vari- :ell lines of alternate origins range .14 μg of BUDR/ml for human adenocarcinoma HeLa to 6.23 μg)R/ml for mouse fibroblast L929. asts L929 and HeLa cells were or 20 and 25 days, respectively, in 2 :UDR/ml with no toxicity.

toxicity of 1 μg of BUDR/ml to 315 and MPC 11 is partially preby 1 μg of thymidine/ml and com- prevented by 10 μg of thymidine/ml. n of 1 μg of thymidine/ml comprotects lymphoma S49.1 from the of 1 μg of BUDR/ml.

sh to thank Dr. Charlotte Friend and Dr. cher for their helpful discussions and critical of this manuscript.

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Received May 10, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 164-167 (1977)

Transmission of Applied Pressure Through Tissues: Interstitial Fluid Pressure, Solid Tissue Pressure, and Total Tissue Pressure (39628)

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When pressure is applied to an area of the skin, the pressure is transmitted through the skin and underlying tissue to structures such as the walls of blood vessels, bone, etc. The effects of applied pressure have been experimentally and theoretically examined as evidenced by the extensive literature discussing the effects of applied pressure on pressure distribution, blood volume shifts, and blood flow rate (cf. 1). However, the effects of external pressure on interstitial fluid pressure and solid tissue pressure in relation to total tissue pressure have not been experimentally examined and only preliminary theoretical effects have been considered (2, 3). In fact, there has been a good deal of confusion because of the failure to distinguish between interstitial fluid pressure and total tissue pressure as evidenced by the fact that the term "tissue pressure" has been used as if only one component of pressure existed in the tissues (cf. 1). The present report uses plethysmography to study the effects of changes in ambient pressure on interstitial fluid pressure, solid tissue pressure, and total tissue pressure in the subcutaneous tissue of the dog hindleg.

Materials and methods. Interstitial fluid pressure (P_{if}) of the dog was measured from capsules implanted in subcutaneous tissue of the upper and lower hindleg 4 to 8 weeks prior to use. (The capsules were a disk-shaped hollow (6 mm x 9 mm i.d.) porous polyethylene matrix (Bolab, Inc.) with an average pore size of 65 μm.) Total tissue pressure (P_{tot}) was measured either with small flaccid balloons inserted subcutaneously or, more frequently, by the needle method for measuring total tissue pressure (see (3) for a discussion of these methods).

Pressure transmission was examined in two different preparations. With the first, the intact hindleg of the pentobarbital anesthetized dog was placed up to the hip in a water filled plethysmograph and P_{if} and P_{tot} measured while applying positive or negative pressure to the leg. With the second preparation, the hindlimb was totally isolated from the dog and artificially perfused with whole blood as described elsewhere (4). The isolated hindlimb was then placed entirely inside the plethysmograph with only the arterial, venous, and pressure measurement catheters extending out of the plethysmograph.

Using the intact hindlimb preparation, the response of interstitial fluid pressure to alterations in ambient pressures was determined as P_{if} was increased above its normal value in the intact hindleg. In order to increase P_{if}, warmed Tyrode's solution was infused intravenously. When P_{if} approached or exceeded atmospheric pressure and the infusion was terminated, P_{if} at first decreased with time as if the tissues were undergoing stress-relaxation. Therefore, measurements were made about 0.5 hr after the infusion of Tyrode's solution when P_{if} appeared to have reached a steady value.

With changes in applied pressure, a small amount of fluid (less than 1 μl) is displaced into or out of the pressure transducer. This fluid shift affected the measured value of P_{if} only during the first several seconds after a pressure change since injection or withdrawal of 1 μl from the capsule was followed by reequilibration in approximately one minute. Therefore, values of P_{if} were taken when P_{if} had appeared to reach a plateau, or about 0.5 to 1.5 min after the pressure change.

Results. *Pressure transmission in intact hindleg with normal hydration.* When pressure in the plethysmograph was varied, total tissue pressure in the subcutaneous spaces of the intact hindleg as measured with both the needle and balloon methods changed by exactly the amount of the change in applied

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e, as expected from a simple me-
il point of view and as would be pre-
from the relative accuracy of the aus-
ry method for determining blood
e. However, in the intact leg, inter-
fluid pressure, as measured by the
:, changed by an average of 71% of
plied pressure when P_{if} was in its
l negative pressure range, irrespective
ther the applied pressure was positive
ative. As illustrated in Fig. 1, with
ressure in the plethysmograph, total
pressure (i.e., the plateau in needle
re) was +1 mm Hg while P_{if} was −6
g. Decreasing external pressure on
; by 10 mm Hg caused total tissue
re to fall 10 mm Hg while P_{if} fell only
Hg. These changes were reversed by
ng external pressure to zero. When
e pressure was applied to the leg,
te pressure changes occurred.

*sure transmission as a function of hy-
i in the intact hindleg.* The effect of
ş the state of hydration of the tissue
ssure transmission was examined in
ɔcutaneous tissues of 9 dogs. Follow-
ntrol measurements, warm Tyrode's
n was infused intravenously. As P_{if}
:ero external pressure) increased, the
tage change in interstitial fluid pres-
in relation to applied pressure) in-
l in every animal. When P_{if} exceeded
n Hg, P_{if} changed by 100% of the
l pressure as seen in Fig. 2. At these
:d P_{if} levels, P_{if} was equal to P_{tot}.

*:t of blood volume shifts on P_{if}
s during pressure transmission in the
l leg.* In another series of experi-
the totally isolated hindlimb was
entirely inside a water-filled plethys-
ph, with only catheters from the ar-
he vein, the capsule, and the needle
ing from the plethysmograph. With
:erial and venous catheters occluded
no change in vascular volume could
P_{if} always changed by 100% of the
l pressure, irrespective of whether P_{if}
ɔsitive or negative. With the femoral
:rs open so that blood could shift into
of the leg, P_{if} changed by an average
4) of 92% of the applied pressure
P_{if} (with no external pressure) aver-
·2.6 mm Hg.

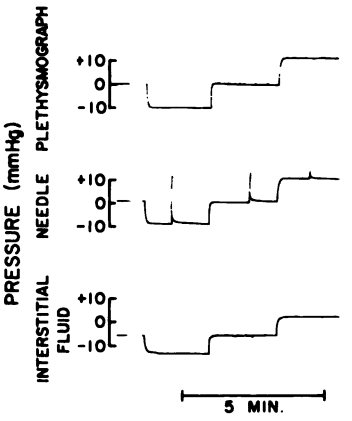

FIG. 1. Examples of the effects of altered external pressure on total tissue pressure (needle pressure) and interstitial fluid pressure.

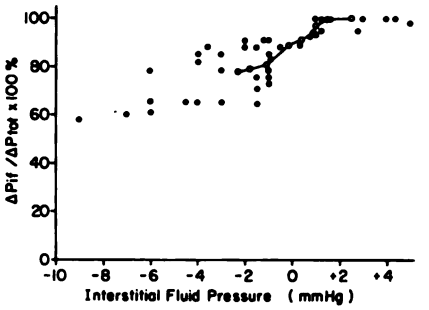

FIG. 2. Summary of pressure transmission to the interstitial fluid (ΔP_{if}) when pressure was applied to the limb surface (ΔP_{tot}) as a function of state of tissue hydration as indicated by interstitial fluid pressure (P_{if}). The solid line connects data from one animal showing the changes in interstitial fluid pressure response (ΔP_{if}) to applied pressure (ΔP_{tot}).

Discussion. This study shows that the interstitial fluid pressure in normally hydrated subcutaneous changes by only 70% of the pressure applied to the skin while total tissue pressure changes by 100% of the applied pressure. In order to interpret these results, it is necessary to familiarize the reader with the current concepts of interstitial structure and to discuss the interrelationships between fluid pressure, solid, pressure, and total pressure in the interstitial spaces.

The interstitium is the extravascular space in tissues that lies between the cells. Its structure is heterogeneous, being composed of fluid elements and nonfluid elements

(collagen, elastin, cell membranes, etc.) (5). Both the fluid and nonfluid elements are independently capable of generating and transmitting pressure. These pressures, i.e., interstitial fluid pressure and solid tissue pressure (P_s) sum to produce total tissue pressure (2, 3):

$$P_{tot} = A_{lf} P_{lf} + A_s P_s,$$

where A is the fraction of the cross-sectional area of the interstitium occupied by fluid (A_{lf}) and nonfluid elements (A_s). The value of total tissue pressure in subcutaneous tissue is determined by the sum of (i) the pressure due to radial tension of the skin and (ii) any external pressure. Total tissue pressure is exactly balanced by the sum of fluid and solid pressures in the interstitium. Obviously, total tissue pressure is responsible for such phenomena as blood vessel compression, while the interstitial fluid pressure participates in regulation of trnascapillary fluid movements.

It may be assumed that the fractional areas, A_{lf} and A_s, do not change with an applied pressure. The change in pressure is then given as:

$$\Delta P_{tot} = A_{lf} \Delta P_{lf} + A_s \Delta A_s.$$

Since (i) A_{lf} and A_s must sum to unity, (ii) both the fluid and nonfluid elements are relatively incompressible, and (iii) neither ΔP_{lf} nor ΔP_s can be greater than ΔP_{tot} if A_s and A_{lf} do no change, the solution to the above equation is $\Delta P_{tot} = \Delta P_s = \Delta P_{lf}$ if the areas do not change. Thus, interstitial fluid pressure as well as solid tissue pressure should change by an amount equivalent to the applied pressure, regardless of the state of hydration. This was found to be true when blood volume shifts were prevented. In the leg where blood volume shifts occurred, P_{lf} changed by less than 100% of the variation in ambient pressure in normally hydrated limbs. It appears that this reduced response may be due to a local tissue distortion which allows A_s and A_{lf} to change so that the amount of pressure carried by the solid elements was increased. For example, consider the effects of applying a pressure of 10 mm Hg to the skin—that is, $\Delta P_{tot} = 10$ mm Hg. If A_{lf} had a constant value of 0.9, then 9 mm Hg of the applied pressure would be carried by the fluid element and 1 mm Hg pressure would be carried by the solid elements. However, the change in fluid pressure would be equal to the change in solid pressure, or $\Delta P_{lf} = \Delta P_s = 10$ mm Hg. On the other hand, in normal tissues (in which blood volume shifts could occur), the data of this study show that ΔP_{lf} would normally be about 7 mm Hg when ΔP_{tot} is 10 mm Hg. Therefore, ΔP_s must be greater than 10 mm Hg and A_s must also have increased.

In overhydrated tissues where P_{lf} was equal to P_{tot}, ΔP_{lf} was found always to equal ΔP_{tot}, presumably because of separation of the solid elements with fluid to such an extent that they no longer make continuous contact so that A_s equaled zero (3).

As yet, there is no way to determine the functional values of A_s and A_{lf}. Because of the lack of experimental determination of these fractional areas, it has been convenient to "surface average" the values of interstitial fluid pressure and solid tissue pressure (3) so that total tissue pressure is expressed as

$$P_{tot} = \bar{P}_s + \bar{P}_{lf},$$

with the bar meaning surface averaged. Also, since A_{lf} is thought to be close to unity (3), the surface averaged interstitial fluid pressure may be approximately equal to the real value of interstitial fluid pressure so that

$$\bar{P}_s = P_{tot} - P_{lf}.$$

This surface averaging and the assumption that A_{lf} is close to unity is necessary in order to allow the functional values to Ps to be determined. The mean values of $\bar{P}_s$ of this

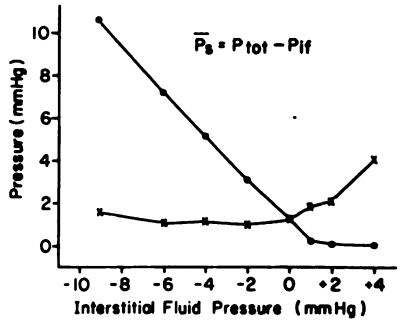

FIG. 3. Averaged measured P_{tot} (×) and calculated $\bar{P}_s$ (○) as a function of P_{lf}.

study are shown in Fig. 3 as a function of P_{if}. Note that P_{tot} changed very little when P_{if} was increased until P_{if} was almost equal to total tissue pressure.

We appreciate the support of NIH Grant HL 11678, the comments and suggestions of Dr. Aubrey Taylor, Dr. Roger Norman, and Dr. Neil Granger, and the excellent secretarial assistance of Mrs. Billie Howard.

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Received May 27, 1976. P.S.E.B.M. 1977, Vol. 15

Evidence for an Inhibitor of Herpes Simp (396

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Introduction. Cells transformed by a virus are often resistant to superinfection by the homologous virus (1). It has been postulated that a specific repressor is responsible for the resistance to superinfection of simian virus 40 (SV40) and adenovirus-transformed cells (2–4). Previous work from this laboratory demonstrated that Herpes simplex virus type 2 (HSV-2) can transform hamster cells (5) and that these cells are resistant to superinfection by HSV-2 (6). In this report, evidence is presented for a soluble inhibitor, obtained from the HSV-2-transformed cells, which inhibits HSV replication in permissive cells.

Materials and methods. Extracts were prepared from normal hamster embryo fibroblast (HEF) cells and HSV-2-transformed HEF cells (333-8-9 and 333-8-9,T-1). The characteristics of the HSV-2-transformed cells have been described (5, 6). These cells maintain virus-specific antigens (5) and contain virus DNA (7). A suspension of 10^8 cells/ml grown in medium 199, supplemented with 10% fetal calf serum (FCS), 10% tryptose phosphate broth, 0.075% $NaHCO_3$, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, was sonicated for 1 min and centrifuged at 100,000*g* for 1 hr. The supernatant was removed and stored at −76° prior to testing for inhibitory activity in plaque reduction assays.

In one type of assay, confluent monolayers of HEF cells were treated simultaneously with 10^2 plaque forming units (PFU) of HSV-1 (strain Patton) or HSV-2 (strain 333), 10 μg of poly-L-ornithine (poly-O; mol wt 120,000), and 0.05 ml of extract. Controls received virus, poly-O, and medium 199, supplemented as described above, instead of extract. After a 1-hr adsorption period, Eagle's medium with 0.5% methyl cellulose, 5% FCS, and 0.225% $NaHCO_3$ was added. Plaques were counted 48 hr postinfection.

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.E I. THE EFFECT OF CELL EXTRACTS ON E FORMATION BY HSV IN HAMSTER CELLS NOT PRETREATED WITH EXTRACT.[a]

ct	Virus	Average number of plaques[b]	Percentage of control[c]
	HSV-1	42	100
	HSV-2	49	100
	HSV-1	TNTC[c]	100
	HSV-2	46	94
T-1	HSV-1	17	41
	HSV-2	21	44

s, extract, and poly-L-ornithine were added to ures simultaneously.
average number of plaques was based on six cultures.
:ntage of control = average number of vith extract/average number of plaques without extract × 100.
xtract was added to these cultures.
numerous to count.

lthough HEF extracts occasionally some reduction in plaque number, nsformed cell extracts consistently much greater reductions. It is possi- t the pretreatment allows the cell to dequately incorporate the inhibitor ıe medium. Since the extracts were d prior to infection in this assay, the apparently does not reduce the num- plaques by inactivating extracellular The extracts could be diluted only l before more than 50% of the activ- lost. Therefore, all further experi- were carried out using undiluted ex-

effect of the cell extracts on replica- HSV-2 was also examined. The data 1 show that the titer of HSV-2 in :lls treated with HEF extract reached 10^4 PFU/ml. The titer of HSV in eated with 333-8-9 or 333-8-9,T-1 fell below the input value and never ed. Thus, the transformed cell ex- lso inhibited replication of HSV.

ıe reduction assays were performed tracts that had been heated at 37 or 30 min before being added to HEF yers. As indicated in Fig. 2, the in- r effect of the transformed cell ex- as lost when extracts were heated to ıt is, the number of plaques in cul- eceiving transformed cell extract at 56° was similar to the number of in cultures with HEF extract. Heating the extracts at 37° for 30 min did not significantly alter the inhibitory activity. The data presented here are for inhibition of HSV-2, but similar results were obtained with HSV-1.

The growth of VSV in HEF cells treated with transformed cell extracts or HEF extract was compared to growth in cells receiving no extract. VSV grew equally well under all conditions employed (data not shown). These experiments indicate that the inhibition of HSV in the transformed cells is not due to classicial interferon, since interferon inhibits VSV and is heat-stable at 56°. The

TABLE II. THE EFFECT OF CELL EXTRACTS ON PLAQUE FORMATION BY HSV IN HAMSTER CELLS PRETREATED WITH EXTRACT.[a]

Extract	Virus	Average number of plaques[b]	Percentage of control[c]
None[d]	HSV-1	72	100
	HSV-2	45	100
HEF	HSV-1	51	71
	HSV-2	31	69
333-8-9	HSV-1	0	0
	HSV-2	1	3
333-8-9, T-1	HSV-1	2	3
	HSV-2	10	21

[a] Extract and poly-L-ornithine were added to HEF cultures 24 hr prior to infection.
[b] The average number of plaques was based on four replicate cultures.
[c] Percentage of control = average number of plaques with extract/average number of plaques without extract × 100.
[d] No extract was added to these cultures.

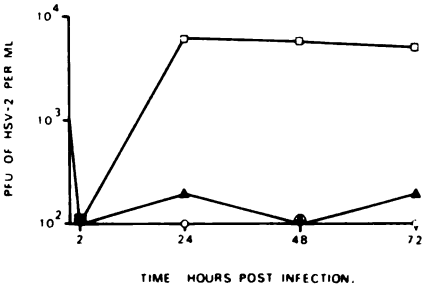

FIG. 1. Replication of HSV-2 (strain 333) in HEF cells continuously treated with cell extracts. Normal HEF cells were treated with cell extracts 24 hr prior to infection with 10^3 PFU of HSV-2. After virus adsorption, extract was readded and replenished every 24 hr. At 2, 24, 48, and 72 hr postinfection, samples were harvested and assayed for infectious virus. (□) Cells with HEF extract; (▲) cells with 333-8-9 extract; (○) cells with 333-8-9, T-1 extract.

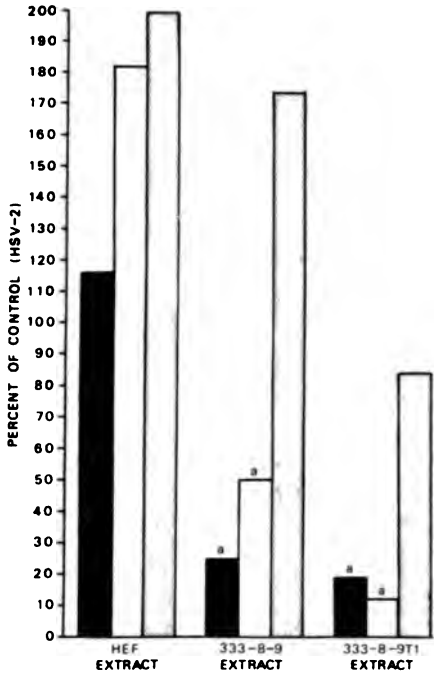

FIG. 2. Detection of inhibitory activity in untreated and heat-treated extracts. Normal HEF cells were exposed to untreated extracts or extracts heated at 37 or 56° for 30 min. After 24 hr, the cells were infected with 10^2 PFU of HSV-2. Plaques were counted 48 hr postinfection. (■) Cells with untreated extract; (□) cells with extract heated at 37°; (▦) cells with extract heated at 56°. Each transformed cell extract was compared to an HEF cell extract under the same temperature conditions by the Student's t test. a Indicates $P > 0.01$

growth of vaccinia virus is also not inhibited by transformed cell extracts (data not shown). The fact that neither VSV nor vaccinia is inhibited by the transformed cell extracts indicates that the extracts are not toxic to viruses.

All experiments were performed at least twice with similar results. The plaque reduction assays, in particular, were repeated four times. The data presented are representative, since different batches of extracts gave levels of inhibition ranging from 60 to 99%. The variability may have been due to the physiological state of the cells when the extract was prepared.

We have previously demonstrated (6) that the HSV-2-transformed cells are resistant to superinfection by HSV-1 and HSV-2 and that the replication cycle of HSV is probably blocked after penetration. The results presented here are consistent with that report. A soluble inhibitor present in HSV-2-transformed cell extracts reduces the plaque formation and growth of HSV in normal cells. The inhibitor probably works intracellularly since the extract can be washed from the cells before infection without precluding inhibition. If this inhibitor acts within the infected or transformed cell to prevent virus production, it is possible that the function of this inhibitor is to regulate the viral genome and, consequently, maintain transformation.

Summary. Extracts of HSV-2-transformed hamster cells inhibit plaque formation of HSV-1 and HSV-2 in normal hamster cells. Extracts also inhibit replication of HSV. The inhibitor is a soluble component of the cells, and probably does not act directly on extracellular virus. The inhibitor is not a classicial interferon since the inhibition is temperature-sensitive and the replication of VSV is not impaired.

Dr. Ronald Duff supplied helpful suggestions for this study. This investigation was supported by Contract NO1 CP 53516 within the Virus Cancer Program of the National Cancer Institute, NIH, PHS.

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Received June 21, 1976. P.S.E.B.M. 1977, Vol. 154.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 171-174 (1977)

ɔcular Response of Human Subjects to Isometric Contraction of Large and Small Muscle Groups (39630)

IE M. RIENDL, ROBERT W. GOTSHALL,[1] JILL A. REINKE, AND JAMES J. SMITH

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liovascular responses to sustained metric) contraction of striated ve been previously described (1- and Horvath (2) reported sharp n both systolic (SP) and diastolic sures and Hymphreys and Lind graded handgrip contractions, rogressive rise in systemic blood BP) and heart rate (HR). From dies of these responses, Lind and oncluded that increases in blood ıd heart rate were comparable for elative tensions in air or leg exer-)ective of the size of the muscle ved (7).

ımonly believed that the pressor static exercise result from a com- f (a) a reflex response initiated by :tion of a humoral agent, perhaps , on sensory endings in contract- (8) and (b) a higher neural "cen- and" factor associated with the effort (9).

:ercise is being used to an increas- for functional evaluation of the system; such tests frequently in- rent-sized muscle masses and var- imental conditions. For example, tory responses in trained subjects uite homogeneous (5) but they le range in the untrained subjects the effect of repetition of static ı the cardiovascular responses has :latively little attention (11, 12). ectives in the current investiga- (1) to study, in untrained normal :cts, the comparative circulatory tatic contraction of a small muscle luctor) and that of a large muscle ntar flexor) and (2) to determine of repetition of these isometric exercises on the cardiovascular responses.

Procedure and methods. Ten normal male subjects (mean age, 25.1 ± 0.7 years) underwent static exercise tests weekly for three successive weeks. All subjects were free of circulatory or other systemic disease as determined by history and physical and cardiovascular examination prior to the study; none of the subjects were athletes or in physical training.

During each test session, static small finger adduction and ankle plantar flexion tests were performed by each subject. In each case, the maximal force output was determined three times (5 min between trials), and the subject was then asked to sustain a contraction equal to 30% of this mean maximal tension to fatigue. The finger adduction was performed first, followed after a 10-min rest by the leg flexion. All the procedures, placements, positioning, and sequences were identical during each of the three weekly visits by the subjects.

A muscle testing table (Keropian Co., San Francisco, Calif., Model 100) was used to indicate the hydraulic isometric force output incident to plantar flexion of the ankle; the table also served to securely support the subject in the sitting position for all tests. A special constructed finger tensiometer served to monitor the adduction force of the little finger and, also, to support the subject's forearm, wrist, and hand. The force output was displayed on a dial visible to the subject and to the operator. To estimate the torque in finger contraction, the tension was multiplied by the distance from the metacarpal-phalangeal joint to the distal interphalangeal joint. From the normal sitting position the knee-raising force exerted by plantar flexion was measured; the force-measuring plate was directly vertical to both the center of the right knee joint and the center of the right ankle joint. The lever arm for

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7 by the Society for Experimental Biology and Medicine d.

ISSN 0037-9727

the leg contraction was taken as the mean distance from the malleoli to the head of the respective ipsilateral metatarsal.

ECG electrodes were placed in a modified Lead II bipolar position. Arterial blood pressure was determined by means of a crystal microphone (Electronics for Medicine Co., White Plains, N.Y.) placed over the right brachial artery under the sphygmomanometer cuff. The pressure in the cuff was altered by an automated inflator-deflator, and the microphone sounds as well as the ECGs were recorded on a Grass Model 7 polygraph. Efforts were made to maintain baseline hemodynamic conditions with a preliminary rest period and the maintenance of a quiet and calm test atmosphere.

Results. The hemodynamic changes are summarized in Table I and indicate significant increases in all variables during isometric contraction in all instances; the mean control SPs and mean ΔSPs (control to peak values) incident to finger and leg contractions were similar. However, sustained contraction of the little finger induced significantly greater ΔDPs (second and third trials) and lesser ΔHRs (all trials) than did leg muscle contraction. The only other difference in circulatory response in the three trials of a single test was a greater control DP in the first trial of finger exercise than in either the second or third trials ($P < 0.01$). There were no differences between recovery values for the circulatory parameters either between the finger and leg contractions or between trials.

Table II indicates the fatigue times and torque outputs for the three trials of the two muscle groups studied. The mean time to fatigue for 30% of maximum voluntary contraction was less for the leg muscle than for the finger in the second and third trials; the

TABLE I. MEAN CIRCULATORY RESPONSES TO STATIC CONTRACTION OF ADDUCTORS OF LITTLE FINGER AND PLANTAR FLEXORS OF ANKLE.[a]

	Finger			Leg		
Trials	1st	2nd	3rd	1st	2nd	3rd
Systolic pressure (mm Hg)						
Control	120.0	122.0	123.8	122.6	124.5	121.8
	±2.2[b]	±3.2	±3.3	±3.5	±3.7	±1.9
ΔSP[c]	29.0	35.0	25.7	33.4	29.0	26.2
	±5.6	±2.8	±4.4	±5.4	±5.5	±4.1
Distolic pressure (mm Hg)						
Control	78.1	74.2	74.1	80.5	80.1	76.4
	±1.32	±1.8	±2.5	±1.6	±4.1	±2.2
ΔDP	28.9	32.3[d]	27.3[d]	23.0	16.9	17.6
	±4.9	±3.4	±3.6	±3.9	±4.8	±3.0
Heart rate (per min)						
Control	72.9	71.9	76.0	72.1	72.7	72.6
	±3.9	±4.3	±2.8	±3.1	±3.9	±3.3
ΔHR	13.7[d]	15.6[d]	11.0[d]	23.3	25.7	18.2
	±3.1	±3.2	±1.6	±4.8	±5.4	±3.2

[a] $N = 10$.
[b] (±) Refers to SE mean.
[c] Δ Values refer to differences from control to peak.
[d] Significantly different from leg values during corresponding trial ($P < 0.05$).

TABLE II. COMPARISON OF FATIGUE TIMES AND TORQUE OUTPUTS DURING STATIC CONTRACTION OF LARGE AND SMALL MUSCLE GROUPS.[a]

	Little finger			Leg		
Trial	1st	2nd	3rd	1st	2nd	3rd
Fatigue time (min)	11.4 ± 4.0	16.8 ± 4.6	15.8 ± 5.0	8.6 ± 1.6	7.2 ± 1.2	6.3 ± 0.8
Torque output (kg-cm)	6.8 ± 0.5	7.8 ± 0.7	8.9 ± 0.7	1576 ± 88	1816 ± 55	1778 ± 106

[a] $N = 10$.

l errors of the mean fatigue times
:, reflecting the large variations seen
arameter. The torque values (Table
ate the much greater output by the
lexors of the foot (about 200-fold).
ıue output of the finger was greater
third trial than on the first ($P <$
nd that of the leg was greater on
second and third trials than on the
< 0.05).

ssion. The marked increases in BP
which occur during static exercise
en noted by other investigators (3–
ulk of the contracting muscle mass
ıpparent effect on the resultant sys-
ssure changes as has also been pre-
eported (7). In our study, the DP
was greater and HR response less
e exercising muscle was smaller; we
ready explanation for these differ-
his divergent effect of the varying
nass on the DP and HR does indi-
wever, a possible dissociation of the
sm for HR and BP changes in static
. Such dissociation was previously
d by the observation that relaxation
ometric handgrip with a tourniquet
results in a prompt fall of HR but
3P (8), and, also, by the lack of
l correlation between individual BP
responses of normal subjects and
patients to static exercise in a pre-
ıdy (13). Although the present in-
on indicates an effect of the size of
ing muscle on the DP and HR
it should be emphasized that, by
predominant factor influencing the
ıamic response is the relative inten-
ıuscle contraction as pointed out by
d McNicol (7). In our study, the
ressures of fatigue were somewhat
i those previously reported (7, 10),
haps, to the unusual experimental
d the employment of muscle groups
ıarily engaged in isometric contrac-

esults indicate that, in spite of a
for an increase in the contractile
erted by the muscle with repetition
ercise, the individual hemodynamic
s to a standard sustained isometric
ion were quite consistent. Thus, it appears that static exercise offers a simple, reliable, and repeatable method for hemodynamic evaluation of the circulatory system.

Summary. Sustained static (isometric) contraction of striated muscle to fatigue was carried out in normal, untrained, young (22 to 29 years) male subjects in order to compare the hemodynamic response of large (ankle plantar flexor) and small (little finger adductor) muscle contraction. There were pronounced increases in systolic and diastolic pressures and heart rates in all cases. The increases in systolic pressure were similar during static contraction of the two different muscle groups; however, diastolic pressure increases were greater and heart rate increases less with finger contraction as compared to leg contraction.

Upon three weekly repetitions, the cardiovascular responses were not significantly altered although the force output was increased with repetition in the case of both muscle groups. The results indicate that (i) although the size of the muscle mass influences the diastolic pressure and heart rate response, the relative muscle tension is the major factor determining the hemodynamic result; (ii) the divergence of blood pressure and heart rate responses suggest a dissociation of the mechanisms governing these two variables in static exercise; and (iii) the relative consistency of the hemodynamic changes during these tests indicates that isometric exercise is a simple and reliable method to study cardiovascular response to stress.

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Received July 20, 1976. P.S.E.B.M. 1977, Vol. 15

ıE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 175-179 (1977)

ıtal Allergic Encephalomyelitis in the Rat: Enhancement and Suppression by Synthetic Polyribonucleotides[1] (39631)

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ɛntal allergic encephalomyelitis ı autoimmune demyelinating dis- central nervous system (CNS) ease can be transferred passively node cells but not with serum ; commonly elicited by intracuta- ction of CNS tissue antigens in Freund's complete adjuvant Recently, complexes of the syn- yribonucleotides, polyadenylic idylic acid (A:U) and polyino- olycytidylic acid (I:C) have been for mycobacteria in CFA and ıt adjuvants were used success- induction of EAE in guinea pigs wever, polyribonucleotide com- ɔften enhance humoral and cellu- y even when injected separately n and without the other compo- FA (6-17). Therefore, attempts here to ascertain whether I:C or ɛnhance EAE even when admin- ıately from antigen and as sole The polyribonucleotide com- also tested for effects on fully EAE and on its passive transfer.

Lewis male rats (Microbiologi- ates, Inc., Walkersville, Md.) 20-380 g were employed. The ıaintained on Purina Laboratory vater. Some rats were bilaterally mized through a mid-dorsal inci- vere given saline as sole fluid

tranded complexes of I:C and ɛither purchased from P-L Bio- nc. (Milwaukee, Wis.) or pre- single stranded polyribonucleo- ; Laboratories, Kankakee, Ill.). ɛr case, equal molar concentra- yadenylic acid (A) and polyuri- dylic acid (U) or polyinosinic acid (I) and polycytidylic acid (C) were incubated together for 1 hr at room temperature. The polyribonucleotides were injected into the penile vein (iv) while the rats were under light ether anesthesia.

An intraperitoneal (ip) injection of 100 mg (wet wt) of rat spinal cord homogenate in saline without any adjuvants constituted a weak encephalitogenic challenge (18), whereas severe EAE was elicited by intracutaneous injection (right foot pad) of 0.05 ml of 40% guinea pig spinal cord homogenate emulsified in an equal volume of CFA. The rats were inspected 8 days after challenge and every day thereafter for clinical signs which include flaccidity of tail, and weakness or paralysis of hindlimbs. All rats were killed 15-18 days after challenge. The entire spinal cords were fixed in Bouin's fluid, embedded longitudinally in paraffin, stained by hematoxylin and eosin, randomized, and scored for histologic EAE lesions.

Passive transfer of EAE was accomplished by injecting rats iv with "EAE cells," obtained from lymph nodes draining sites of inoculation in donors that had been immunized 8 days previously in the right foot pad with 0.05 ml of guinea pig spinal cord homogenate emulsified in CFA (19). A donor:recipient ratio of 1:16 constituted a weak challenge whereas a ratio of 1:1 or 1:2 was a strong challenge. The rats were examined for clinical signs daily. When it was desirable to elicit histological EAE lesions within 24 hr after the injection of EAE cells, a localized form of passive transfer was employed. Acceleration and localization of the EAE lesions was induced by inflicting a thermal injury in the right cerebral hemisphere of the recipients 2 days before the transfer (19). The rats were sacrificed 1 day after the cell transfer. The brains were fixed, embedded, sectioned coronally,

wholly by a grant from the Kroc Foun-

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ISSN 0037-9727

and stained as above, and the slides were randomized and scored for EAE lesions localized near the region of the thermal necrosis.

Results. One iv injection of 0.6 mg of I:C, administered at the time of a weak encephalitogenic challenge, resulted in a moderate but significant increase ($P < 0.05$) in the number of rats that had clinical and histological manifestations of EAE, whereas administration of 0.6 mg of A:U or of I alone or C alone had no effect (Table I). I:C treated rats also had more extensive histological lesions than controls; this difference barely failed to reach statistical significance when only animals with lesions were analyzed ($P > 0.05$), but it was significant when all rats were included ($P < 0.01$). The adjuvant effects of I:C were only observed when the encephalitogenic stimulus was weak (i.e., 100 mg of rat spinal cord ip); pilot experiments have shown that an increase of the challenge dose to 200 mg of cord increased the incidence and severity of EAE and administration of I:C did not cause further enhancement.

The mechanism by which I:C enhances immunization against a weak encephalitogenic stimulus was investigated with the aid of the passive transfer system. The ability of polyribonucleotides to affect the activity of fully immunized EAE cells in the rapid (1-day) localized passive transfer system was studied first. The results were the opposite of those obtained with active immunization. I:C, administered to recipients on the day of cell transfer, greatly reduced the numbers of localized EAE lesions if the donor:recipient ratio was 1:1 and completely prevented the formation of localized lesions if the donor:recipient ratio was 1:16 (Table II). Both Miles Laboratories and P-L Biochemicals preparations of I:C were effective. The suppression of localized EAE was not due to nonspecific adrenal-mediated stress effects inasmuch as I:C was also inhibitory in adrenalectomized recipient rats. Since I:C is quite toxic for adrenalectomized rats (20), only small quantities of I:C (0.06 or 0.3 mg) were used in this experiment. These small doses were equally effective in intact and adrenalectomized recipients (Table II). Other ribonucleotides were not effective; A:U on the day of EAE transfer was much

ABLE II. EFFECTS OF SYNTHETIC POLYRIBONUCLEOTIDES ON PASSIVELY TRANSFERRED LOCALIZED EAE.

Recipients and donor:recipient	Treatment of recipients	Time[a]	Histologic scores (individual rats)[b]
Intact 1:1	I:C, 0.6 mg	DO	1 1 1 1 1 1 1 0 0 0 0 0
		D-1	4 3 1
		D-6	4 4 4
	A:U, 0.6 mg	DO	4 3 3 3 3 3
	I, 0.6 mg	DO	4 4 4 4 4 4
	C, 0.6 mg	DO	4 4 4 4
	Saline (control)	DO	4 4 4 4 4
Intact 1:16	I:C, 0.6 mg	DO	0 0 0
		D-1	1 0 0
		D-6	2 1 1
	Saline (control)	DO	2 1 1
Adrenex[c] 1:2	I:C, 0.3 mg	DO	2 2 2
Intact 1:2		DO	2 1 0
Adrenex 1:2	I:C, 0.06 mg	DO	2 2 1 0 0 0 0
Intact 1:2		DO	2 2 2
Adrenex 1:2	Saline (control)	DO	4 3 3 3
Intact 1:2		DO	4 4 3

)O = day of passive transfer of EAE cells. D-6 and D-1 refer to 6 and 1 days before passive transfer.
cored from 0 to 4+ according to number and extent of perivascular inflammatory lesions localized adjacent
: zone of thermal brain necrosis.
bilateral adrenalectomy 3 days before cell transfer.

BLE III. EFFECTS OF I:C-CONTAINING BLOOD SERUM ON PASSIVELY TRANSFERRED, LOCALIZED EAE.

Treatment of recipients[a]	Histologic scores (individual rats)[b]
:	4, 4, 4, 4, 4, 3
l from I:C-treated rats	1, 0, 0, 0, 0, 0, 0
n from I:C-treated rats; serum ubated with saline	0, 0, 0, 0
n from I:C-treated rats; serum ubated with RNase	4, 4, 4, 3
n from untreated rats; serum ubated with RNase	4, 4, 4, 3

All recipients received EAE cells at a donor:recipient ratio of 1:1. All treatments were 4 ml in ne.
cored from 0 to 4+ according to number and t of perivascular inflammatory lesions localized ent to the zone of thermal brain necrosis.

nanently destroyed or only temporarily pressed the activities of fully immunized E cells. Therefore, I:C was tested in the localized passive transfer system where e is a 4-day latent period between administration of EAE cells and the develop- t of clinical signs. I:C, 0.6 mg, was administered iv to recipients 1 day after they received the passive transfer of EAE s. The disease was not significantly al- d; the four I:C-treated recipients devel- d clinical signs 5 or 6 days after passive sfer and they all became paralyzed, e the four saline-treated controls developed signs 4 to 6 days after transfer and two became paralyzed and two became weak. This indicates that the suppressive effects of I:C on the fully immunized EAE cells were transient and nondestructive.

In spite of the transient nature of the inhibition of fully immunized EAE cells by I:C, we hoped that these short-term effects could be utilized to treat EAE after clinical signs had appeared. EAE was, therefore, produced in 21 rats by active immunization with guinea pig spinal cord and CFA in the footpad. At the time that clinical symptoms first appeared (11 days after challenge), the rats were divided into three equal groups which were treated iv once with 0.6 mg of I:C or with A:U or with saline. None of the treatments changed the course of the illness. In each group there were five or six deaths after nearly equal survival periods (3.0, 3.4, 2.7 days, average, respectively) after treatment. Apparently, the effects of I:C were too weak or too transitory to have any effect at such a late stage of the disease process.

Discussion. Gumbiner *et al.* (4) and Paterson (5) have demonstrated that guinea pigs injected intracutaneously with guinea pig spinal cord and Freund's adjuvant developed EAE if either mycobacterial RNA, A:U, or I:C were included in the emulsion. Paterson (23) speculated that the adjuvant effects of these double-stranded polyribonu-

cleotides rested on their ability to activate macrophages and to expand the numbers of specific thymus-derived lymphocytes which can be sensitized by myelin basic protein.

The present experiments differed from Gumbiner *et al.* (4) and Paterson (5, 23) in that we used rats instead of guinea pigs, an aqueous encephalitogenic antigen without adjuvants instead of an emulsion in CFA, and a separate iv injection of polyribonucleotide instead of an admixture with antigen. Nevertheless, we also observed enhancement with I:C, but unlike these authors, there was no enhancement with A:U. Recently, Paterson (24) reported that even in the absence of CNS antigens, Freund's incomplete adjuvant containing either A:U or A was able to induce EAE lesions in the choroid plexus and neuraxis of guinea pigs. There is no evidence that I:C can elicit this phenomenon or that it can occur in rats. Nevertheless, we verified the nonencephalitogenicity of I:C under the conditions of these experiments by injecting 0.6 mg iv into each of five rats and observing them for 17 days. There were no clinical signs or histologic lesions of EAE.

A number of experimental designs have demonstrated that I:C and A:U can enhance cellular immunity and delayed hypersensitivity (4, 5, 10, 12–17). However, in other experimental designs, I:C has been shown to suppress cell mediated immune responses (11, 25–28). As far as we know, the present study is the first one to demonstrate the ability of I:C to enhance and to suppress, respectively, the initiation and the expression of the same delayed hypersensitivity syndrome. Enhancement of cell-mediated immune responses by I:C has often been attributed to the ability of I:C to increase the proliferation of thymus-derived lymphocytes (4, 5, 17) and/or to stimulation or activation of macrophages (5, 14–17). Suppression of cell-mediated immune responses by I:C has been blamed on its cytotoxicity (11, 25–28); I:C can cause a leukopenia and lymphopenia in the peripheral circulation (29, 30); depletion of lymphoid tissues has also been described (31). In the present study, too, depletion of lymphocytes from thymus-dependent areas of the spleen occurred regularly in recipients of a

y I:C was not mediated by the adre-
d or by induction of interferon. The
tly opposite effects of I:C on active
sive production of EAE may be ex-
by its biphasic effects on lympho-
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Received September 21, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 180–183 (1977)

Dithiothreitol Augments Renin Activity by an Action on Renin Substrate[1] (39632)

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Renin is not generally believed to be a sulfhydryl-dependent enzyme. Thus hog renin was not inhibited by *p*-chloromercuribenzenesulfonic acid or *N*-ethylmaleimide (NEM) which block free SH-groups (1). Furthermore, treatment of renin with dithiothreitol (DTT, Cleland's reagent) did not influence its pressor potency, as determined by direct assay in the rat (1). Hog renin was also reported to be resistant to treatment with *p*-chloromercuribenzoate (PCMB) and iodoacetamide (2). Human renin acting on hog substrate has similar properties since it was not inhibited by iodoacetamide, merthiolate, or phenylmercuriacetate (3). However, Waldhausl and Lewandowski (3) reported that human renin is greatly potentiated by DTT and dithioerythritol (DTE), and suggested that the results with DTT indicated that unprotected SH-groups existed within the active center of the enzyme. These conflicting results and the uncertainty in the literature on the importance of SH-groups in the renin–angiotensin reaction led us to investigate the effects of DTT on bovine renin. Preliminary studies had indicated that DTT potentiates the reaction of bovine renin with homologous substrate but not with tetradecapeptide substrate (TDP). The present work shows that DTT has different effects on the renin–angiotensin reaction depending on the substrate utilized. In addition, the evidence indicates that the potentiating effect of DTT on angiotensin generation is due to an effect on the substrate and not on the enzyme.

Methods. Preparation of bovine serum substrate. Substrate was prepared according to the method of Lever *et al.* (4). The final preparation was precipitated between 1.5 and 2.3 *M* ammonium sulfate and dialyzed against PO_4 buffer (150 m*M*, pH 7.0) containing EDTA (10 m*M*). The material was then frozen in 1.0-ml aliquots.

Preparation of bovine renin. Bovine kidneys were obtained from a local slaughterhouse. The cortex was separated and frozen. After thawing, 100-g lots were homogenized in a Waring Blendor with 1 liter of H_2O. Further treatment, to be described in detail elsewhere, included acid treatment, ammonium sulfate fractionation, batch adsorption, and elution from DEAE cellulose and from kaolin, and finally acetone treatment.

Renin assay. Bovine renin was incubated for 30 min at 37° with bovine or hog substrate (200 pmole/ml) in a volume of 250 μl. The final concentration of PO_4 was 75 m*M* and of EDTA was 5.0 m*M*. DTT or NEM was added at the beginning of the incubation in some experiments. After incubation, 2.0 ml of Tris buffer (100 m*M*, pH 7.4) was added and the tubes were placed on ice. All assays were done in duplicate or triplicate. Aliquots of 50 μl were used for radioimmunoassay of angiotensin I. Renin assays were linear with enzyme concentration within the range of samples used.

Pretreatment experiments. In some experiments, either substrate or enzyme was preincubated for 10 min at 37° with DTT and then diluted with H_2O to lower the DTT concentration. Aliquots of the pretreated enzyme or substrate were then used in the final reaction mixture at the usual conditions.

Radioimmunoassay. Angiotensin I was assayed by the radioimmunoassay technique of Haber *et al.* (5), scaled down to a 200-μl volume. The isotope, antibody, and standards were obtained from New England Nuclear. The range of standards was from 25 to 1600 pg. The supernatant after charcoal

[1] Supported by grants from the Kansas Heart Association, the American Heart Association, and NIH HL 16102.

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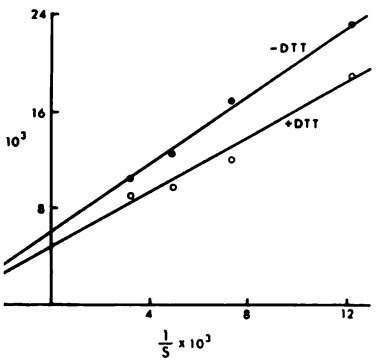
ISSN 0037-9727

t was added to 5.0 ml of Aquasol ıted in a Packard Tri-Carb liquid on counter.

ıls. Hog substrate was obtained es Laboratories; DEAE-cellulose, ıd DTT from Sigma Chemical Co.; ɔm Fisher Scientific.

. Effect of DTT on the kinetics of -angiotensin reaction. Bovine renin ted with increasing concentrations ɛ substrate in the presence or ab- DTT (10 m*M*). Angiotensin gener- s potentiated by the presence of ıe results, plotted according to the ɔf Lineweaver and Burk, show that reases the V_{max} without changing ig. 1). When a similar experiment : with hog substrate, the potentiat- : of DTT was much more dramatic. ıe V_{max} increased while the K_m re- ınchanged (Fig. 2).

ɪtment of renin and substrate with ıce the effect of DTT was much ɛctive on hog substrate than on bo- ɪtrate and since other experiments wn) did not indicate any effect of the reaction with tetradecapeptide , it seemed likely that the effect of ; exerted on the substrate and not enzyme. Therefore, experiments ied out in which the enzyme or the was pretreated with DTT (to re- -groups) and then diluted so that



Effect of dithiothreitol (DTT) on the reac- :n bovine renin and bovine substrate. Line- rk plot of the results in the absence or pres- T (10 m*M*). Ordinate: $1/V \times 10^3$, where *V* l in micrograms of angiotensin per hour per Abscissa: $1/S \times 10^3$, where *S* is expressed in ɔer milliliter.

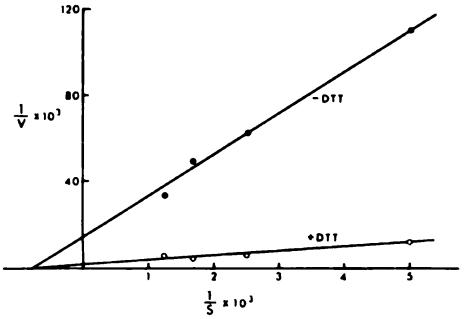


FIG. 2. Effect of DTT (10 m*M*) on the reaction between bovine renin and hog substrate. Lineweaver-Burk plot with units expressed as Fig. 1.

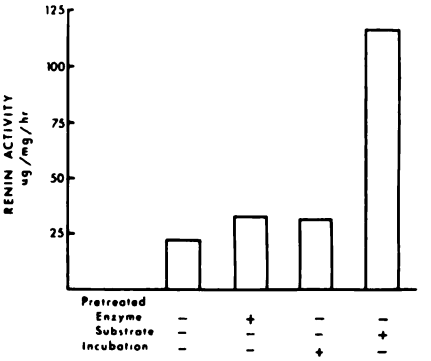


FIG. 3. Effect of DTT pretreatment on the reaction between bovine renin and hog substrate. Renin or substrate was preincubated with DTT (10 m*M*) and then diluted so that the final DTT concentration was 0.3 m*M*. Pretreatment of the enzyme (column 2) caused a slight increase in renin activity compared to the control (column 1), while pretreatment of the substrate caused a large increase (column 4). Addition of DTT (0.3 m*M*) during the final incubation (column 3) (no preincubation) caused a slight increase in renin activity. Renin activity is expressed as micrograms of angiotensin per milligram of protein per hour.

the concentration of DTT in the final assay mixture was low. Pretreatment of the enzyme with DTT (10 m*M*) followed by dilution so that the final DTT concentration during incubation was 0.3 m*M* produced a slight increase in renin activity, but this did not exceed the value obtained with 0.3 m*M* DTT present only during the incubation (Fig. 3). However, pretreatment of the substrate (DTT, 10 m*M*) followed by dilution to the same final DTT concentration (0.3 m*M*) produced a striking potentiation (Fig. 3).

Effect of NEM. Since DTT is still present in the incubation mixture after pretreatment of the enzyme and dilution, the small potentiating effect could still have been exerted through an effect on the substrate. To test for this possibility, use was made of NEM, which reacts with free SH-groups (as in DTT) and does not inhibit renin (1). Addition of NEM (5.0 m*M*) to the final incubation reaction did not affect the renin activity, while DTT (0.5 m*M*) produced a twofold potentiation. However, when both NEM and DTT were present, there was no potentiation, indicating that NEM had reduced the concentration of DTT to an ineffective value (Fig. 4). When hog substrate was pretreated with DTT (10 mM) and then diluted to yield a final DDT concentration of 0.5 m*M*, there was a dramatic potentiation (sevenfold). Addition of NEM (5.0 m*M*) to this reaction mixture, at a concentration shown to completely block the potentiating effect of 0.5 m*M* DTT, caused a slight reduction in angiotensin generation. The decrease was similar to the decrease caused by the addition of NEM to the reaction mixture containing 0.5 m*M* DTT (no preincubation) (Fig. 4). Therefore, in this condition (excess NEM present) there was probably little free DTT during the reaction and yet the dramatic potentiation due to DTT pretreatment was evident. Since the enzyme in this experiment had not been exposed to an effective concentration of DTT, the augmented renin activity can only be due to an effect of DTT on the substrate.

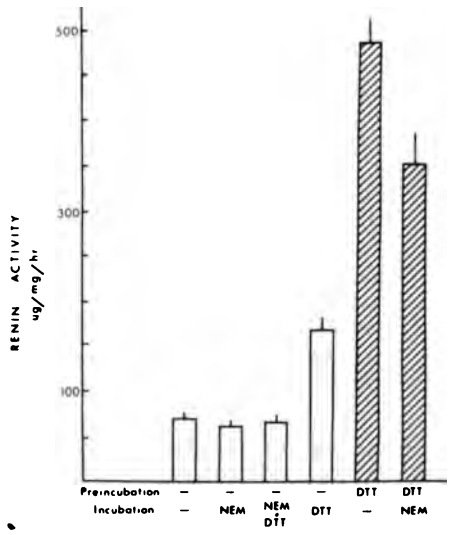


FIG. 4. Effect of NEM and DTT on reaction between bovine renin and hog substrate. When NEM was used, it was present during the final incubation at 5.0 m*M*. DTT was either added to the final incubation at 0.5 m*M* or preincubated at 10 m*M* for 10 min with the substrate followed by dilution so that the final concentration was 0.5 m*M*. Renin activity is expressed as in Fig. 3. The enzyme preparation used in this experiment was from a different batch from that used in Fig. 3 and had a specific activity about 2.5 times greater.

Discussion. The present study shows that dithiothreitol increases the amount of angiotensin generated by bovine renin acting on either bovine or hog substrate. The kinetic analysis suggests that this phenomenon is not due to an increased affinity of the enzyme for the substrate. Since the V_{max} was increased, it is possible that DTT reduced disulfide bonds or protected sulfhydryl groups either in the enzyme or in the substrate, which are important for the catalytic reaction. However, the preincubation experiments showed clearly that the effect was exerted on the substrate. Even when the effective DTT concentration was rendered negligible by the presence of an excess of NEM during the incubation, the influence of DTT pretreatment of the substrate on angiotensin generation was striking (Fig. 4). The concentration of DTT employed (10 m*M*) was chosen because Waldhausl and Lewandowski reported that higher concentrations caused gelation of their incubation mixture and they showed results with 10 m*M* DTT (Table 3 in Ref. 3) in comparison with other activators and inhibitors of the renin-substrate reaction.

These results may help clarify the inconsistent reports in the literature on the importance of SH-groups for renin activity. Since there are consistent reports that treatment of renin with organic mercurials or NEM does not inhibit its activity, this should have been a clue that renin is not a sulfhydryl-dependent enzyme. In fact, studies by Haas *et al.* with PCMB, iodoacetate, and cysteine led them to conclude, "A reactive thiol group is probably not involved directly in the catalytic activity of renin" (6). Their studies on the sulfhydryl groups of renin did suggest, however, that while the enzyme

vas fully active in its reduced or oxidized tate, it was more susceptible to denaturaion in the reduced state (6). Therefore, the ong-term influence of reduced sulfhydryl roups on renin and on its substrate may ave opposite effects on the final generation f angiotensin.

It seems likely that renin substrates from arious species contain SH-groups which are mportant for the renin–angiotensin reacion. Depending on the different methods of reparation and the history of the substrate rior to its use, there may be different mounts of free SH-groups at the time the eaction is studied. Thus, the apparent disrepancy concerning the role of SH-groups n renin is probably due to variations in the ubstrate and not the enzyme.

Summary. Dithiothreitol (DTT) increases he generation of angiotensin when bovine enin acts on bovine substrate or hog subtrate. Kinetic analysis indicates that the V_{max} increases without a change in K_m. Prereatment of bovine renin with DTT followed by dilution of the DTT to a low conentration does not augment renin activity, while pretreatment of the substrate and a similar dilution reveals marked potentiation. *N*-Ethylmaleimide (NEM) does not inhibit renin activity but does prevent the augmenting effect of DTT present during the incubation. It is concluded that DTT augments renin activity by an action on renin substrate and that renin does not fall in the category of sulfhydryl-dependent enzymes.

We acknowledge the valuable technical assistance of Mr. Paul Arnold and Mrs. Roselle Poisner.

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Received May 25, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 184–187 (1977)

Localization in Rat Skin Transplants of Purified ^{125}I-Labeled Xenogeneic Histocompatibility Antibody (39633)

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It has been known for some time that properly prepared xenogeneic antisera can distinguish between different major histocompatibility antigens (1). We have now found it possible to prepare, from two different types of xenogeneic antisera, ^{125}I-labeled histocompatibility antibody able to localize *in vivo* with a high degree of specificity in rat skin grafts where only the transplanted skin carries the corresponding antigen. One source of such antisera was from rabbits immunized with a fraction prepared from a tumor originating and carried in Fischer-344 (F-344) rats. A second source was a commercially available horse anti-rat lymphocyte sera (Microbiological Associates, Bethesda, Maryland, Lot. No. 131182).

It was also found that ip injections of ml amounts of this unfractionated horse serum resulted in a high degree of blocking of the localization in such skin grafts of either the labeled antibody prepared from the immune horse serum or a labeled histocompatibility antibody prepared from the sera of allogeneically immunized rats. The rapidity and completeness of this blocking suggests that its cause was the preempting by antibody from the unlabeled sera of antigen sites where the labeled antibody would otherwise have localized.

Materials and methods. Preparation of allogeneic antibody. The general methods used in preparing ^{125}I-labeled antibody to the strong histocompatibility antigen expressed on F-344 rat erythrocytes have already been described (2, 3). Briefly, Buffalo strain rats were immunized by repeated transplants of a tumor induced by methylcholanthrene in an F-344 rat. From such immune sera, γ-globulin was isolated and labeled with ^{125}I. Labeled antibody was isolated by a twice repeated procedure that consisted of the absorption of the antibody onto F-344 erythrocytes, followed by the elution of absorbed antibody from ghosts prepared from these cells. Recently, we have obtained much higher yields of antibody by partially purifying the antibody before labeling.

Preparation of ^{125}I-labeled rabbit antibody. Rabbits were immunized with a "lipoprotein" fraction rich in histocompatibility antigens from the same F-344 carried tumor using a modification (4) of the procedure developed by Manson *et al.* (6). Immunization consisted of four weekly injections of "lipoprotein," each of 6 mg protein content, in complete Freund adjuvant, divided between two im sites. Antisera was obtained 10 days after the last immunization. The γ-globulin isolated from the immune sera was further purified by absorption and elution from additional tumor lipoprotein, labeled with ^{125}I, and *in vivo* absorbed by iv injection into Buffalo strain rats which were bled 2 days later. Antibody was isolated from the sera by absorption on F-344 red cells and elution at pH 3.2 from ghosts prepared from these cells. *In vitro* incubation of a sample of the final product showed 47% of the ^{125}I-label binding to F-344 red cells, 1.1% to Buffalo red cells.

Preparation of ^{125}I-labeled horse antibody. Four-milligram portions of γ-globulin prepared from horse anti-rat lymphocyte sera were labeled with ^{125}I, then *in vivo* absorbed by iv injection into Buffalo rats. From sera obtained 2 days later by heart puncture labeled antibody was separated by absorbing onto F-344 erythrocytes, and elutions from ghosts were prepared from these erythrocytes. In one experiment, from 6.6 mCi of ^{125}I attached to a total of 1.78 mg of horse γ-globulin injected into two rats, 4.9% was recovered in 6 ml of rat sera, and 0.13 μCi as purified antibody. *In vitro* this antibody bound 43% to F-344 red blood cells and 1.6% to Buffalo erythrocytes.

Preparation of ^{131}I-labeled normal Buffalo

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ISSN 0037-9727

ulin. After labeling 4 mg of normal lo γ-globulin prepared by ammonium e precipitation, the labeled material μCi/rat) was injected iv into normal lo rats. Three days later the rats were ınd the serum was stored frozen until in double-label experiments.

vivo studies. Skin grafts were per-:d as described by Billingham and Sil-(7) with slight modifications. Small grafts weighing less than 0.4 g were :d of fat and loose connective tissue. Buffalo rat, approximately 150 g in t, received a Buffalo skin isograft on ft side as a control for an F-344 skin on the right side.

:s were given drinking water 625 μ*M* from 3 days after skin transplantation, njected with a paired dose of ^{125}I-la-antibody and ^{131}I-labeled normal γ-lin 4 days after the transplant. One ater the rats were heparinized and by severing the vena cava. Fluothane-ıs used for anesthesia during all proce-. Radioactivity measurements were with an automatic well-type scintilla-:ounter. Variability of results is indi-as the standard deviation of individual ; from the mean.

ults. A substantial number of experi-; have been carried out showing that a ed, ^{125}I-labeled alloantibody to a ; F-344 histocompatibility antigen will ze preferentially in F-344 skin grafts ıffalo rats. Some of these results have been published (4, 5). More recent experiments have also shown that prior administration of horse anti-rat lymphocyte sera will largely prevent such localization. Table I shows the results of one such experiment. Horse anti-rat lymphocyte sera was administered ip, in 0.5-ml doses, 2 days before skin transplant, on the day of skin transplant, and 4 days later, on the day of labeled antibody injection. Rat weights averaged 190 g and F-344 skin grafts, 0.31 g, or 0.163% of the average rat weight. The ^{125}I skin values of Table I are computed from values that averaged 18.16% of the injected ^{125}I dose found in the F-344 grafts on Buffalo strain rats that did not receive horse antiserum and 3.51% found in rats that received 3 injections of this horse antisera.

In this experiment the dose of ^{125}I-labeled antibody was 13,696 cpm and of ^{131}I-labeled normal γ-globulin, 14,402 cpm. The ^{125}I content of F-344 skin grafts in rats receiving no horse antiserum averaged 24,292 counts per 10 min (cp10m) above an average background of 97 cp10m. The net ^{131}I count was 562 cp10m. In the rats receiving horse antiserum the F-344 skin grafts averaged a net 4817 cp10m for ^{125}I and 346 cp10m for ^{131}I.

Figure 1 shows the results of a similar experiment in which the ^{125}I label was attached to a histocompatibility antibody prepared from the serum of one rabbit immunized with a fraction prepared from F-344 rat tissue. In this experiment the rat weight averaged 167 g, and the F-344 skin trans-

ABLE I. CONCENTRATIONS OF ^{125}I-LABELED RAT HISTOCOMPATIBILITY ANTIBODY AND ^{131}I-LABELED NORMAL RAT γ-GLOBULIN IN BUFFALO RATS, SOME BEARING F-344 AND BUFFALO SKIN GRAFTS.[a]

	Percentage injected dose in tissue equal to 1% rat weight (mean ± SD)							
	No horse anti-RL sera				3 doses horse anti-RL sera			
	Four grafted rats		Average of two normal rats		Four grafted rats		Average of two normal rats	
Tissue	^{125}I	^{131}I	^{125}I	^{131}I	^{125}I	^{131}I	^{125}I	^{131}I
	2.29 ± 0.71	4.61 ± 0.38	4.76	5.24	2.39 ± 0.48	3.65 ± 0.47	3.44	3.78
	3.73 ± 1.20	7.75 ± 0.43	7.95	8.72	3.68 ± 0.51	5.80 ± 0.59	5.99	6.45
ıin graft	1.23 ± 0.51	1.69 ± 0.30			1.09 ± 0.58	1.25 ± 0.26		
n graft	111.3 ± 47.8	2.27 ± 0.50			21.54 ± 5.38	1.44 ± 0.33		
uffalo skin	0.47 ± 0.15	0.60 ± 0.11	0.42	0.36	0.47 ± 0.05	0.46 ± 0.05	0.50	0.55
	0.97 ± 0.31	1.68 ± 0.33	1.88	1.92	0.90 ± 0.15	1.31 ± 0.13	1.31	1.30
	0.46 ± 0.12	0.72 ± 0.07	0.82	0.74	0.46 ± 0.08	0.66 ± 0.14	0.72	0.72
	0.70 ± 0.24	0.95 ± 0.11	1.24	1.15	0.67 ± 0.10	0.77 ± 0.12	0.88	0.90
	0.61 ± 0.18	1.10 ± 0.09	1.12	1.40	0.65 ± 0.11	0.94 ± 0.11	0.83	1.00

; were injected iv, 4 days after making skin transplants, with a paired mixture of ^{125}I-labeled antibody to a major F-344 histocompatibility id ^{131}I-labeled normal Buffalo γ-globulin, then killed 24 hr later. Horse anti-rat lymphocyte (horse anti-RL) sera was given, in 0.5-ml doses, ip, fore skin transplant, on the day of skin transplant, and just before labeled antibody injection.

plants, 0.187 g. The ^{125}I skin values of Fig. 1 are calculated from an average of 9.0% of the injected dose found in the F-344 grafts. These rats received no horse serum injections. The ^{125}I dose was 7982 cpm, the ^{131}I dose 6928 cpm. The F-344 skin grafts averaged a net 7157 cp10m for ^{125}I and 149 cp10m for ^{131}I.

Table II shows the results of an experiment similar to the experiment reported in Table I except that the ^{125}I label was attached to a histocompatibility antibody isolated from horse anti-rat lymphocyte sera. This experiment clearly shows localization

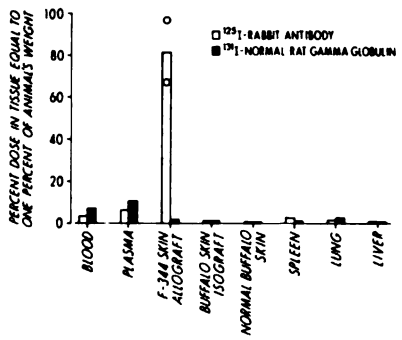

FIG. 1. Distribution of ^{125}I-labeled rabbit antibody recognizing F-344 rat histocompatibility antigen and of ^{131}I-labeled normal rat γ-globulin in Buffalo strain rats carrying transplants of F-344 and Buffalo skin. Rats were injected 4 days after the skin transplants were made, and killed 24 hr later. Values are the average for 2 rats. O, individual rat values.

of the ^{125}I-antibody in the F-344 sk on Buffalo rats and no obvious bi Buffalo strain tissue. Administratio mune horse serum, given, as ip dos ml, 6 and 4 days before labeled injection, and on the day of injectio this case also blocked antibody loca The ^{125}I concentration in the F-3 grafts is lower by a factor of 10 receiving the horse serum.

In this experiment the net ^{125}I d 6493 cpm, the ^{131}I dose, 6442 cpm not receiving horse antiserum th skin grafts averaged a net 4782 cp ^{125}I and 301 cp10m for ^{131}I. In rats r horse antiserum the F-344 skin gra age a net 442 cp10m for ^{125}I and 18 for ^{131}I.

Since the labeled antibody of Tab prepared from horse γ-globulin, or ble explanation or partial explana this blocking action on antibody loc was that the horse antiserum in over the 6-day period, had induce mune response to horse γ-globulin some way blocked the labeled anti calization. Therefore, an additional ment was carried out similar to ported in Table II except that the tration of immune horse serum was to two injections of 0.5 ml each g day preceding and the day of label body injection. The labeled antibo localized with high specificity in th

TABLE II. DISTRIBUTION OF ^{125}I-LABELED HORSE XENOANTIBODY AND ^{131}I-LABELED NORMAL γ-GLOBULIN IN BUFFALO RATS CARRYING F-344 AND BUFFALO SKIN GRAFTS.[a]

	Percentage injected dose in tissue equal to 1% rat weight (mean			
	Group 1 (No horse anti-RL sera)		Group 2 (three doses hors sera)	
Tissue	^{125}I	^{131}I	^{125}I	
Blood	2.01 ± 0.13	5.26 ± 0.43	1.32 ± 0.24	3.90
Plasma	3.08 ± 0.24	8.48 ± 0.52	1.85 ± 0.33	6.09
Buffalo skin graft	0.91 ± 0.19	1.34 ± 0.40	0.43 ± 0.34	1.33
F-344 skin graft	41.9 ± 6.0	2.68 ± 0.66	4.15 ± 1.11	1.72
Normal Buffalo skin	0.29 ± 0.07	0.62 ± 0.06	0.38 ± 0.09	0.49
Lung	0.93 ± 0.18	1.87 ± 0.21	0.60 ± 0.17	1.40
Liver	0.41 ± 0.07	0.82 ± 0.07	0.33 ± 0.08	0.59
Spleen	0.82 ± 0.12	0.81 ± 0.13	0.40 ± 0.09	0.55
Kidney	0.53 ± 0.07	1.17 ± 0.15	0.36 ± 0.06	0.94

[a] Each group, with and without injection of 0.5-ml doses of horse anti-rat lymphocyte (horse anti-RL) a 6-day period, consists of four rats. All rats were injected iv, 4 days after receiving skin transplants, with of ^{125}I-labeled horse antibody reacting with an F-344 strong histocompatibility antigen and ^{131}I-label Buffalo γ-globulin. Rats were killed 24 hr after injection.

afts on otherwise untreated Buffalo
he administration of the unlabeled
ntiserum reduced this localization by
r of 6.7.

ussion. The experimental results re-
here show that it is possible to pre-
rom xenogeneic antisera produced in
or horses, labeled histocompatibility
ly able to localize after iv injection
high degree of selectivity in rat skin
vhere only the skin transplant carries
responding antigen. The reason for
sence of the histocompatibility anti-
1 the horse anti-rat lymphocyte sera
s to some extent a matter of specula-
resumably the rat cells, or a portion
at cells used for the horse immuniza-
rried a strong histocompatibility anti-
it is expressed in F-344 rats.

most likely cause of the rapid block-
horse antiserum of labeled antibody
ition in target tissues is the saturation
gen sites in the skin transplant by
mpatibility antibody present in the
ntiserum. This effect might be sup-
ited by an antibody induced reduc-
the number of available antigen sites,
ct termed antigenic modulation by
d Boyse and their co-workers (8, 9).
he horse anti-rat lymphocyte serum is
powerful immunosuppressant, and
ght also affect antibody localization,
stulated mechanisms for the blocking
would be better established if experi-
using unlabeled histocompatibility
ly isolated from horse antiserum
l a similar blocking action.

discovery of the blocking action of
se antisera was due to experiments in
he immunosuppressive effects of this
erum was used, by the technique of
(10), to permit the growth in Buffalo
ats of tumors induced by methylchol-
ne in F-344 rats. It turned out that
ition of labeled histocompatibility an-
in these tumors was much lower than
und in experiments that were identi-
ept that immunosuppression was in-
by whole body X irradiation plus cor-
injections.

milar antibodies are present in anti-
lymphocyte sera, a possibility would
o exist that the survival of transplants
an kidney and other organs in pa-
tients treated by anti-lymphocyte sera might sometimes be influenced by the presence of this type of antibody.

Summary. It is shown that appropriate xenogeneic rabbit and horse antiserum, as well as allogeneic rat serum, can serve as sources from which ^{125}I-labeled antibody to a strong histocompatibility antigen expressed on Fischer-344 strain rat cells can be prepared. After iv administration all three types of antibody would localize preferentially in F-344 skin grafts made 4 days earlier on Buffalo strain rats. After 24 hr, ^{125}I localization ranged from 10 to 20% of the injected dose on F-344 skin grafts averaging 0.3 g weight on 150–190-g Buffalo rats. The horse serum used as an antibody source was a commercially available anti-rat-lymphocyte serum. Prior ip administration of two or three doses of 0.5 ml of this serum largely blocked uptake by F-344 skin grafts of both the ^{125}I-labeled alloantibody and the ^{125}I-labeled xenogeneic histocompatibility antibody prepared from this horse antiserum.

This work was supported by USPHS Research Grant CA-16749 from the National Cancer Institute and under contract with the U.S. Energy Research and Development Administration with the University of Rochester and has been assigned Report No. UR-3492-913.

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Received May 26, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 188-191 (1977)

Effect of Carbon Tetrachloride on Hepatic Transport of Ouabain in Developing Rats (39634)

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Newborn rats are immature in their ability to excrete drugs by the liver. For example, Klaassen (1) observed that the biliary excretion of sulfobromophthalein (BSP) is low in newborn rats, resulting in plasma concentrations of BSP that are five times higher in newborns than in adults. Similarly, hepatic excretion of ouabain is low in young rats, and this is considered to be responsible for the high toxicity of ouabain in immature rats (2).

Although young rats have a low capacity to excrete drugs by the liver, hepatic excretory function may be enhanced in these animals by hepatic microsomal enzyme stimulators such as phenobarbital, pregnenolone-16α-carbonitrile, and spironolactone (3). These agents also enhance biliary function in adult rats, but the stimulation observed in adults is qualitatively different from the effect in young rats (3). The enhancement of biliary function in adult rats involves increases in bile flow and transport of drugs (such as ouabain) from liver to bile. Thus, stimulation with these agents results in reduced concentrations of ouabain in plasma and liver. In contrast, the principle effect of these microsomal enzyme stimulators in young rats is to increase the transport of ouabain from plasma into liver such that young animals have less ouabain in plasma but higher concentrations in liver (3). These results suggest that stimulation of the hepatic excretory mechanism is possible at two sites within the hepatocyte; enhancing uptake (sinusoidal) and secretion (canalicular).

It was of interest to determine whether the "site" of altered biliary function following acute liver damage was also age dependent. Carbon tetrachloride (CCl_4) is an agent widely used to produce experimental liver damage in laboratory animals (4) and biliary function is depressed following CCl_4 poisoning as indicated by retention in plasma of drugs administered secondarily. The effect of CCl_4 on hepatic drug transport however is not specific. Altered transport of both anions (5-8) and neutral compounds (9) by the liver has been demonstrated following CCl_4 although these compounds are transported by separate mechanisms (10).

Liver slices and isolated perfused livers from CCl_4-treated adult rats showed no differences in BSP uptake, but biliary excretion of BSP was decreased following CCl_4 (5). Retention of BSP in plasma following CCl_4 was primarily due to decreased biliary excretion associated with decreased bile flow (6). Ouabain (neutral compound) retention in plasma was accompanied by accumulation of the drug in the liver (9). These results are consistent in that CCl_4 appears to have a specific effect on the transport of drugs from liver to bile and does not appear to alter the uptake of drugs from plasma into liver.

However, CCl_4 treatment may result in a reduction in BSP uptake in isolated perfused rat livers when measured soon after poisoning (7). Furthermore, a decrease in the maximal removal rate of indocyanine green (ICG) from plasma has been observed following CCl_4 where hepatic uptake was exclusively measured (8). Thus, the results suggest that liver damage may be quantitated at two sites within the hepatocyte.

The effect of CCl_4 on hepatic transport may be age dependent and the purpose of studies described in this paper was to determine the effect of CCl_4 on ouabain transport in developing rats. The hepatic transport of ouabain was chosen as the index of biliary function because ouabain is known to be actively transported by rat liver, is not metabolized appreciably in the rat (11), and is transported to a lesser extent following CCl_4 (9).

Materials and methods. Lactating female rats with litters of eight male offspring

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ISSN 0037-9727

tan Research Animals, Inc., Haslett,) were housed in shoe box cages and allowed food and water *ad libitum*. ring were separated from mothers at 4 ; of age.

s of 14, 20, 24, 32, and 45 days of age ed a single ip injection of corn oil (5) or CCl_4 (1 ml/kg) prepared in corn his dose of CCl_4 is known to alter / function in adult rats (6, 9). The iistration of CCl_4 across litters was ar- d so that both control and CCl_4- d animals were obtained from each Twenty-four hours following adminis- n of CCl_4, animals were lightly anes- ed with ether and injected with [^{3}H]- in (1 mg/kg) via the tail vein. The ic activity of [^{3}H]ouabain was 250 ng and was prepared by mixing nonra- tive ouabain (Sigma Chemical Co.) randomly labeled [^{3}H]ouabain (New nd Nuclear). Thirty minutes following iistration of ouabain, rats were anes- ed with ether, a blood sample was (with a heparinized syringe) by car- uncture, and the entire liver and small ine were rapidly removed. The 30-min period was chosen to compare the ef- f CCl_4 in developing animals directly previous reports on the effect in adults

ce young animals are too small for col- n of bile, the amount of radioactivity intestine was measured to estimate / excretion of ouabain (3). The intes- as homogenized in 5 or 10 ml of water 200-μl aliquot of whole homogenate dded to 15 ml of Dimilume counting on (Packard Instrument Co.). Samples r (100 mg) and plasma (200 μl) were l in scintillation counting vials and sol- ed overnight at 40° with 1 ml of So- -100 (Packard Instrument Co.) before on of 15 ml of Dimilume counting on. Radioactivity in all samples was ated with a Packard Model 3380 liquid lation spectrometer equipped with au- ic external standard for quench cor- n.

tistical analysis of data were made by sis of variance (12) and the level of icance was chosen as $P < 0.05$.

ults. The effect of CCl_4 on hepatic transport of ouabain in rats of various ages is shown in Fig. 1. CCl_4 treatment resulted in significantly higher plasma concentrations of ouabain at every age tested. Coincident with the retention of ouabain in plasma, CCl_4-treated animals excreted less ouabain

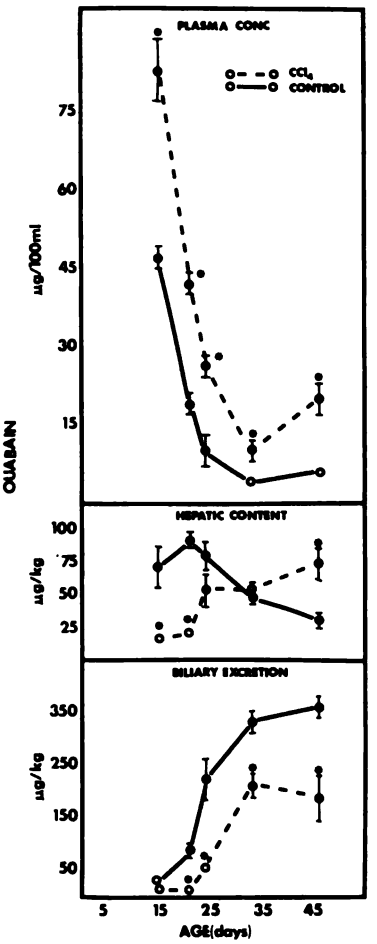

FIG. 1. Rats of ages ranging from 14 to 45 days were injected ip with corn oil or CCl_4 (1 ml/kg) prepared in corn oil. After 24 hr, rats were injected with [^{3}H]ouabain (1 mg/kg) via the tail vein. Thirty minutes following ouabain, animals were sacrificed following cardiac puncture, and plasma, liver, and intestine were analyzed for tritium. Hepatic and intestinal ouabain content are factored by kilograms body weight. Each point represents the mean (± SE) of five to eight rats obtained from two or three litters. When standard error bars are not shown, standard error was smaller than the diameter of the point. * indicates that values are significantly different from corresponding control ($P < 0.05$).

into bile. The lower amount of intestinal (biliary) ouabain was significant except in 15-day-old animals.

Of particular interest is the effect of CCl_4 on hepatic concentration of ouabain. In young animals (less than 25 days of age), CCl_4 treatment resulted in significantly less ouabain in the liver, suggesting impaired uptake. In contrast, 46-day-old CCl_4-treated animals had significantly more ouabain in the liver. Inasmuch as the 46-day-old group retained ouabain in plasma and excreted less into the intestine, the high concentration of ouabain in liver is due to impaired transfer of ouabain from liver to bile.

Although ouabain was retained in plasma of 25- and 33-day-old treated rats and less intestinal ouabain was detected in these animals, no significant differences were observed in the hepatic concentration of ouabain (Fig. 1).

Discussion. The major effect of CCl_4 on hepatic transport of ouabain in adult rats was a decrease in the transport of ouabain from liver to bile (9). The data presented here are consistent with this conclusion in that the CCl_4-induced retention of ouabain in plasma was accompanied by accumulation of the drug in liver in 46-day-old rats (Fig. 1). This effect, however, appears to be age dependent. Indeed, treated rats younger than 25 days of age could not effectively accumulate ouabain from plasma into liver (Fig. 1).

From the data presented in this study it would be difficult to ascertain the mechanism for the age difference. One possibility may be that CCl_4 acts at a different "site" on the parenchymal cell in young rats than in adults. This would appear to be true, since the effect of CCl_4 on hepatic concentration of ouabain is exactly opposite in rats of different ages.

Since it is believed that CCl_4 toxicity is mediated through a toxic metabolite (13–15) and young animals are immature in their ability to metabolize drugs (16, 17), the toxicity of CCl_4 may be expected to be age dependent. From this explanation, however, one might expect a difference in the dose of CCl_4 required to elicit toxicity but not in the "site" of damage. It is important to note that the dose of CCl_4 used in this study (1 ml/kg) was enough to cause retention of ouabain in plasma but did not result in death to animals at any age tested. Furthermore, although newborn (1-day-old) rats are resistant to CCl_4 toxicity, Dawkins (18) observed that the 7-day-old rat appeared to be as sensitive as the adult to the hepatotoxic action of CCl_4. Since the youngest rat used in this study was 15 days old, the age difference in the qualitative effect of CCl_4 on ouabain transport was probably not due to differences in the extent of CCl_4 toxicity.

In adults, CCl_4-induced drug retention in plasma could be partly due to decreased hepatic uptake. Indeed, for organic anions (e.g., BSP and ICG), depressed uptake was observed following CCl_4 (7, 8). However, BSP and ouabain retention in plasma (in adults) is thought to be mainly due to decreased hepatic secretion into bile (6, 9). Nevertheless, CCl_4-induced hepatic damage is not specific and appears to affect both sinusoidal and canalicular transport depending upon the experimental design or the test compound used. Retention of ICG in plasma following CCl_4 was due to depressed uptake under experimental conditions designed to measure only uptake (8). Furthermore, in adult rats, the rate-limiting step in BSP transport is hepatic secretion, not uptake (19, 20) which accounts for the observation that retention of BSP in plasma following CCl_4 was primarily due to decreased biliary excretion (6). Similarly, in adult rats, hepatic extraction of ouabain is six times that for BSP (21) which would make detection of an alteration in ouabain uptake following CCl_4 unlikely.

The results of this investigation demonstrate that the effect of CCl_4 in young rats is qualitatively different from the effect observed in adults. These results are similar to observations made by Klaassen (3) on the effect of hepatic stimulators on ouabain transport in developing rats. Since the biological responses (liver damage with CCl_4 vs hepatic microsomal enzyme stimulation with phenobarbital) induced with these chemicals are markedly different, age may be an important variable in characterizing the effect of drugs on biliary function. That is, like CCl_4, phenobarbital may affect the

cular correlate of both hepatic uptake
›iliary secretion of drugs in all ages, but
levelopmental status of the animals is
rtant in determining the *in vivo* charac-
ıtion of the response. The present re-
suggest that at some time during post-
development (approximately 33 days)
: is a shift in the rate-limiting step in the
tic transport of drugs and that experi-
al treatments may simply act to exag-
e age differences that already exist.

mmary. CCl_4 depressed hepatic excre-
function in adult and developing rats.
iis regard, plasma ouabain concentra-
were significantly higher and biliary
ɜtinal) ouabain was significantly lower
ɜ treated animals. Of particular interest
the observation that hepatic ouabain
ignificantly lower than controls in CCl_4
ɜd young rats (decreased uptake into
), but significantly higher than controls
ɜated older rats (decreased secretion
liver into bile). These results suggest
the "site" of altered biliary function
wing CCl_4-induced liver damage is age
ndent.

s investigation was supported in part by Na-
Institute of Health Grant ES00560. S.Z.C. was
rted by a predoctoral fellowship from the Eli
Co. The authors acknowledge the technical
nce of Ms. Harriett Sherman.

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Received July 20, 1976. P.S.E.B.M. 1977, Vol. 154.

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Granulocytopoiesis: Studies on Leukocytosis-Inducing and Colony-Stimula Factors (39635)[1, 2]

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Introduction. Two humoral agents, neutrophilic leukocytosis-inducing factor (LIF)[3] and colony-stimulating factor (CSF)[4] are believed to assist in maintaining normal granulocyte levels in the blood and marrow (1, 2). This concept is based on the cyclic fluctuation seen in the blood neutrophil level of some normal individuals and the presence of a large reservoir of mature neutrophilic cells in the marrow (1-3). According to this concept, when the blood neutrophil level reaches the low normal, LIF is released which accelerates the mobilization of mature neutrophils from the marrow reserve into the blood. In addition, reduction in blood neutrophil level is considered to result in release of CSF which is believed to stimulate proliferation and differentiation of the stem cells, myeloblasts, promyelocytes and myelocytes in the marrow (1, 2, 4-6). This concept is quite attractive and is supported by studies suggesting that LIF is a different moiety from CSF (4, 7).

The purpose of the current studies was to clarify further the relationship between leukocytosis-inducing factor and colony-stimulating factor activities.

Methods. Peritoneal lavages. Four healthy mongrel dogs weighing 15-20 kg were splenectomized in order to exclude marg and demargination of granulocytes the peritoneal lavage and LIF testing dures. Two months or more after tl gery and when their hemograms we mal, the dogs were subjected to per lavages under strict aseptic condition scribed for peritoneal dialysis (8). A ml of blood was obtained for total a ferential leukocyte counts and a mar pirate was taken for differential co described below, 2 g of sterile potato in 1000 ml of warm normal saline fused into the peritoneal cavity. Fron of the dogs, a fraction (~200 ml) infusate was withdrawn immediately beled as control lavage fluid. Eight later, blood and marrow samples wer taken for the purposes stated abov peritoneal fluid was collected into blo lecting bags and was labeled as lavage cavity was washed with 3-4 liter of normal saline. At the end of washing potato starch in 1000 ml of warm saline was infused. Eight and eighteer later, the above procedure was re (except that potato starch was not i after the last lavage), and the lavage labeled as II and III. Total and diffe leukocyte counts were done on the l and washes as on the blood sample washes were discarded. The lavage centrifuged at 1500*g* for 15 min to cell-free fluid. All fluids were cultu aerobic and anaerobic bacteria. The were filtered using 0.45 μm Nalge and frozen at −20°C. From these sa CSF and LIF activities were deter Before assaying the fluids for CSF an limulus assay (9) was done on sample two dogs to detect endotoxin cont tion.

[1] This work was supported by the U. S. Energy Research and Development Administration, NIH Research Grant No. CA-08888, and American Cancer Society Grant No. DT-5.

[2] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[3] LIF refers to neutrophilic leukocytosis-inducing factor activity. It is also known as neutrophil-releasing factor, neutrophilia-inducing factor, and leukocytosis-inducing factor.

[4] CSF refers to the ability of a sample to stimulate proliferation of granulocytic-macrophage colonies from target marrow cells *in vitro* in soft agar medium.

[5] Sybrom Corporation, Rochester, N.Y.

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and CSF are believed to be α-globu-
0, 11). To determine whether a corre-
exists between the levels of LIF and
with α-globulins, the α-globulins as
s the total protein in the lavage fluids
quantitated by electrophoresis on cel-
acetate strips.

idling of blood and marrow samples. blood leukocyte counts were obtained he Coulter Model F counter, and dif-ial counts, by counting 200 cells on t-Giemsa stained smears. Marrow nu-d cell differential counts were done by ng 500 cells on Wright-Giemsa d smears.

ct of Escherichia coli endotoxin on the neutrophils. After three of the eight fluids tested by limulus assay were to contain endotoxin in concentra-ıp to 0.3 ng/ml, a study was done to nine whether the amounts of endo-present in the lavage fluids injected for say were responsible for the observed jection blood neutrophil changes. *richia coli* endotoxin,[6] at a dose of 28 g for two dogs and 84 ng/dog for three was injected iv in 10 ml of normal in 1–2 min. Blood samples (1 ml were obtained from these dogs imme-before and after the injection and at 1, 6, 12, and 24 hr after the injection. and differential leukocyte counts were ed from these samples as noted

in the lavage fluid samples. Approxi-6 weeks after lavage, the lavage were tested for LIF activity in the dog which they were obtained, with at least ks allowed to elapse between testing ferent fluids. Each lavage fluid was as follows. After 1 ml of blood was ed for total and differential leukocyte , a lavage fluid (5 ml/kg body wt) was d iv in 5 min. At 1, 2, 4, 6, 12, and 24 r the injection, blood samples (1 ml were obtained for total and differen-ıkocyte counts. The maximum eleva-the blood neutrophilic band and seg-d forms obtained after the injection of , expressed as the percentage of the immediate preinjection value, was taken as the LIF activity of the fluid.

CSF in the lavage fluids. CSF activity in the lavage fluids was determined *in vitro* by the method of Marsh *et al.* (12). Homologous marrow cells, 2×10^5 per plate and three–five plates per sample, were used as target cells. A group of ≥17 cells was defined as a colony.

Lavage fluids from two dogs were dialyzed against distilled water for 48 hr to exclude the presence of inhibitors (13). Both dialyzed and nondialyzed samples from these dogs were tested simultaneously for CSF activity.

Results. Cells removed in lavages and granulocytic cellularity in the blood and marrow during lavages. The numbers of cells removed from the peritoneal lavages of the four dogs are shown in Fig. 1; also shown are the blood and marrow neutrophil changes noted before, during, and for 2 days after lavages. The numbers of total cells and of various types of cells removed were about the same in the three lavages. Total band plus segmented forms and monocytes removed at each lavage ranged from 11.4 to 16.8 $\times 10^9$ and 1.8 to 2.7 $\times 10^9$, respectively.

Blood band forms were 140/mm³ at basal level and were 860, 640, and 630/mm³ at lavages I, II, and III, respectively. The segmented forms were 5.5×10^3 at basal level and were 17.0, 13, and 9.5×10^3/mm³ at lavages I, II, and III, respectively. Both band and segmented forms were significantly ($P < 0.05$) greater at lavages I, II, and III than at the basal level. At 20 and 40 hr postlavage III, band forms were not significantly ($P > 0.1$) different from those at the basal level.

The marrow M:E ratio (Fig. 1) was significantly ($P < 0.05$) higher at lavages II and III and 20 hr later than at the basal level. The subsequent value was not significantly ($P > 0.1$) different from the basal value. Sequentially, the myeloblasts + promyelocytes and the myelocytes increased in the marrow as the M:E ratio increased. These cells were significantly ($P < 0.05$) higher at lavage III than at the basal level, but, at other points of analysis, they were not significantly ($P > 0.05$) higher. Metamyelo-

rthington Biochemical Corporation, Freehold,

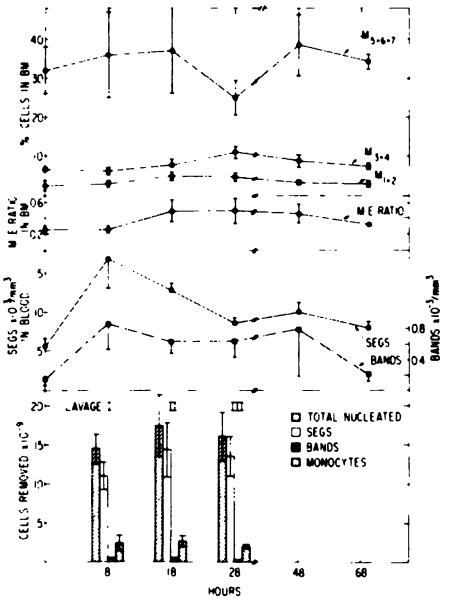

FIG. 1. The results of four studies plotted against time after the first infusion of starch + saline into peritoneal cavities of dogs; mean and SEM are shown. Bottom section: numbers of various types of cells recovered from lavages. Second section from bottom: blood neutrophilic bands and segments. Second section from top: M:E ratio of bone marrow (BM), with M representing myeloblasts + promyelocytes + myelocytes and E, all stages of normoblasts + proerythroblasts + erythroblasts. Top section: marrow differential counts as percent of total nucleated cells. M_1 = myeloblast, M_2 = promyelocyte, M_3 = large myelocyte (> 17.0 μm in diameter), M_4 = small myelocyte (< 16.9 μm in diameter), M_5 = metamyelocyte, M_6 = band form, and M_7 = segmented form.

cytes + band forms + segmented forms decreased to 0.78 times the basal value at lavage III; approximately 20 hr later, they were 1.2 times the basal value, and, in the subsequent sample, they decreased toward the basal value; however, neither the decreased nor the increased value was significantly different from the basal value. Marrow spicules from the aspirates obtained at lavage III and later showed increased granulocytic cellularity.

LIF in the lavage fluids. The pattern of changes in the levels of band and segmented forms in the blood of the dogs following the injection of lavages I, II, or III was similar. Their levels were elevated at 1 or 2 hr (the first determination), and peak levels were reached at 4 to 6 hr after the injection.

LIF activity of the lavage fluids is shown in Fig. 2. In the control lavages it was 34% above the basal level. It was maximum in lavage I, 237%, and showed a slight but insignificant ($P > 0.25$ for lavages I vs II and for I vs III) reduction in lavages II, 199%, and III, 213%. LIF activity in control lavage fluid was different ($P \leq 0.01$) from that in lavage I, II, or III.

Blood neutrophil changes after endotoxin injection. Changes in the blood neutrophilic band and segmented forms of dogs injected with 28 or 84 ng of endotoxin (Table I) were less than those found after control lavage injection. The maximum increase in mean number of band and segmented forms was 21.5% above the basal value after the larger of the two doses of endotoxin was administered.

CSF in lavage fluids. Addition of control lavage fluid to culture plates resulted in production of an average of 11.8 colonies per plate (Fig. 2). The plates with no added stimulus grew ≤10 colonies per plate. The maximum CSF activity, found in lavage I, induced proliferation of 50.8 colonies per plate (430% of the control), compared with 22.2 (188% of the control) and 17.3 (146% of the control) for lavages II and III, respectively. The CSF activity in lavage I was significantly different from that in lavages II ($P > 0.005$ and <0.01) and III ($P < 0.005$). The activity in lavage II was not significantly

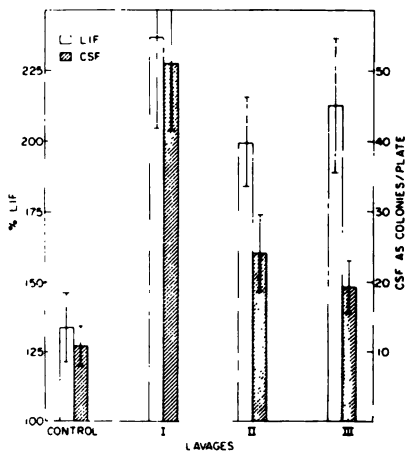

FIG. 2. Results (mean and SEM) of four studies (three for control). LIF activity is maximum elevation of band + segmented forms in the blood after injection of lavage fluid (usually occurring at 4–6 hr), expressed as the percentage of immediate preinjection value. CSF activity is the number of colonies per plate (see text).

.E I. NEUTROPHILIC BAND AND SEGMENTED FORMS EXPRESSED AS PERCENTAGE (MEAN AND SEM) OF PREINJECTION VALUE IN THE BLOOD OF DOGS INJECTED WITH *E. coli* ENDOTOXIN.

toxin injected (ng/dog)	Immediate Preinjection	Immediate postinjection	Hours after injection of endotoxin					
			1	2	4	6	12	24
	100.0	102.0	79.0	69.0	76.0	85.0	82.0	93.0
hree dogs)	±0.0	±6.5	±3.0	±7.0	±1.4	±9.0	±2.0	±27.9
	100.0	100.3	96.3	93.3	115.3	112.3	121.5	107.0
wo dogs)	±0.0	±4.4	±1.4	±7.5	±13.3	±19.3	±14.3	±4.1

ent from that in lavage III ($P > 0.2$), he activity in lavage III was only y significantly different from that in ntrol ($P = 0.05$). There was no corre- ($P > 0.25$) between the LIF and CSF y levels of lavage fluids II and III, ting that the CSF and LIF are two t entities.

amount (average of two-dog studies) + α_2 globulins was 5, 157, 128, and 57 and the total protein was 40, 824, nd 297 mg% in the control lavage and ges I, II, and III, respectively. Corre- of α-globulins or total protein with vas good ($P < 0.05$), but with LIF it oor ($P > 0.25$).

CSF activity was similar in the di- l and nondialyzed samples. Results of tudy are shown in Table II. These s indicate that there was no dialyzable tor in any of the lavages.

cussion. Induction of peritoneal in- ation with potato starch and removal utrophils from the peritoneal cavities gs in this study were associated with mobilization of neutrophilic band and nted forms from the marrow into the . Similar rapid mobilization of cells he marrow into the blood with lavag- s been shown in rats (14). The lavage dure was also associated with in- d granulocytopoiesis in the marrow as ted by the increase in the marrow M:E the percentage of M_{1-4} cells, and the locytic cellularity.

induction of inflammation and lavag- the peritoneum were also associated he release of LIF into the peritoneal . The LIF level was probably elevated blood of these dogs as well as in the fluids, since plasma samples obtained rats during peritoneal leukopheresis ned increased LIF levels (15). Injec- f the lavages into the dogs from which

TABLE II. CSF ACTIVITY (MEAN AND SEM OF THREE PLATES) IN DIALYZED AND NONDIALYZED FLUIDS.

Fluid	Lavage number		
	I	II	III
Nondialyzed	128.7 ±7.4	87.7 ±2.9	44.0 ±2.0
Dialyzed	130.0 ±2.8	86.7 ±5.8	50.6 ±1.67

they were obtained induced elevation in the number of blood neutrophilic band and segmented forms, which indicated rapid mobilization of the neutrophils from the marrow into the blood. Persistence of the elevated LIF activity in all three lavages was not unexpected in view of the sustained demand for the cells in the periphery with continued peritoneal inflammation and lavaging.

The LIF activity demonstrated in the lavages was not due to bacterial contamination since only sterile fluids were tested. Further, the observed LIF activity in the lavages was not due to endotoxin for the following reasons. (i) Blood neutrophil level was already increased in the first blood sample (1–2 hr) obtained after injection of the lavage fluids. It is known that after an endotoxin injection the blood neutrophil level drops for 2–4 hr with a subsequent increase (16). (ii) A maximum of 0.3 ng of endotoxin/ml of lavage fluid was estimated by limulus assay in three out of eight fluids tested before injection into dogs for LIF assay. At this contamination level, the total amount of endotoxin injected into each dog was 30 ng. Injection of 84 ng of E. coli endotoxin/dog in this study (Table I) or as much as 100 ng of *Salmonella abortus equi* endotoxin/dog by others (16) did not perceptibly increase the blood neutrophil count. (iii) In addition, LIF-activity of those lavages with endotoxin (as recognized by limulus test) was not different from the ac-

tivity of those with no detectable contamination.

CSF was also released into the peritoneal cavity in response to the inflammation of the peritoneum. The CSF activity in the lavages was not due to endotoxin contamination, since addition of endotoxin directly to the culture plates did not stimulate colony formation (17). The reasons for the precipitous reduction of CSF activity between lavages I and II are not clear, but the possibilities include rapid catabolism of the produced CSF, exhaustion of CSF stores from the CSF-producing tissue, masking of the CSF by inhibitors, and/or rapid utilization by the target cells. Evidence indicating that the CSF binds to those cells capable of forming granulocytic-macrophage colonies (CFU-C) in *in vitro* cultures (18) supports the last of these possibilities. Masking of the inhibitors was not likely since dialysis of the fluids against water did not reveal the presence of inhibitors; however, the other possibilities cannot be excluded.

The temporal relationship between the reduction in CSF in lavages II and III and increasing M:E ratio and percentage of M_{1-4} cells in the marrow of these dogs suggests that the CSF is probably inducing the M_{1-4} cells to proliferate. CSF has been shown to be essential for the persistence and proliferation of CFU-C in *in vitro* cultures, and, as noted above, it binds to CFU-C (18). The marrow CFU-C are increased in adult mice injected with concentrated human urinary CSF (19). These observations suggest that CSF is influencing granulocytopoiesis *in vivo* by acting on CFU-C and M_{1-4} cells, as has been suggested by other investigators (4, 5). Whether the CSF acts on pluripotent hemopoietic stem cells is not clear.

The nonconcordant levels of LIF and CSF activities demonstrated in lavage fluids II and III indicate that LIF and CSF are two distinct entities. A similar suggestion has been made by others who noted evidence of increased LIF activity and relatively less elevated CSF in the plasma of rats and mice given repeated doses of endotoxin (4, 7). Both LIF and CSF are reported to be α-globulins (10, 11), and CSF has been demonstrated to constitute only a small fraction of the total serum α-globulins (20). In view of these observations it is likely that both LIF and CSF constitute only a small fraction of the total α-globulins in the lavage fluids. Without additional studies on purified preparations, the good correlations observed in lavage fluids between CSF and α-globulins and the lack of such correlation between LIF and α-globulins can be considered only as additional evidence suggesting that the activities of CSF and LIF are mediated by separate moieties.

Summary. Three peritoneal lavages at intervals of approximately 8–10 hr were performed in each of four splenectomized dogs. Induction of peritoneal inflammation and removal of neutrophilic cells from the peritoneal cavity was associated with a rapid mobilization of the neutrophilic band and segmented forms from the marrow into the blood and increased granulocytopoiesis in the marrow. The fluids removed from the peritoneal cavity contained both neutrophilic leukocytosis-inducing factor (LIF) and colony-stimulating factor (CSF) activities. The intensity of blood neutrophilia noted in dogs following the iv injection of fluids was considered to reflect LIF activity. CSF activity was assayed by the capacity of the lavage fluids to stimulate dogs' marrow cells to form granulocytic-macrophage colonies *in vitro* in soft agar medium. The increased LIF activity level was observed in lavages I, II, and III to approximately the same extent. This observation indicated that the production of LIF, having the function of mobilizing mature neutrophils from the marrow into the blood, continued as long as the peripheral demand for the cells existed. The CSF activity was maximum in lavage I and was precipitously reduced in lavages II and III. Following the increase of CSF level in lavages, the marrow M:E ratio, the percentage of myeloblasts + promyelocytes + myelocytes, and the granulocytic cellularity increased, which suggested that the CSF was influencing marrow granulocytopoiesis.

The pattern of fluctuation of the CSF activity in the lavages was different from that of the LIF activity, which indicated that CSF and LIF are two different entities.

We acknowledge the technical help of Ms. Louise Honikel and Mr. James Lehmann.

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Received July 28, 1976. P.S.E.B.M. 1977, Vol. 154.

Effect of Somatostatin on Insulin Secretion Ir

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Somatostatin, a hypothalamic peptide initially reported to suppress pituitary growth hormone secretion (1), has been found to possess extra-pituitary effects. The secretions of gastrointestinal hormones such as gastrin (2) and secretin (3) have been reported to be inhibited by somatostatin as have the release of the pancreatic glucoregulatory hormones, insulin (4), and glucagon (5). The ability of somatostatin to inhibit hormone secretion in various endocrine tissues has prompted the suggestion that this peptide may act on a common step in secretory processes (6).

Calcium ion is recognized as an essential requirement for the secretion of most, if not all, peptide hormones (7) and recent evidence suggests that somatostatin's inhibition of glucose-induced insulin release may be related to the divalent cation (8, 9). In an effort to explore the relationship between somatostatin and calcium further, we have investigated the inhibitory action of somatostatin on glucose and ionophore A23187-induced insulin secretions in the isolated, perfused dog pancreas.

Methods and materials. Pancreases were obtained from mongrel dogs (17–25 kg) and perfused according to procedures previously described (10). Normal perfusate consisted of Krebs–Ringer bicarbonate buffer containing 4% dextran and 75 mg% glucose. The ionic concentrations of this medium in mEq/liter are: Na^+, 143; K^+, 5.9; Ca^{2+}, 2.5; $H_2PO_4^-$, 1.2; Mg^{2+}, 1.2; Cl^-, 125.8; SO_4^{2-}, 1.2; and HCO_3^-, 24.6. Cyclic somatostatin (Bachem, Inc.) and ionophore A23187 (generously supplied by Dr. Robert Hamill, Eli Lilly and Co.) were added as required. A23187 was dissolved in ethanol before being added to the desired perfusate (11).

[1] Supported in part by NIH Grant No. 5 SO1-RR-05350.

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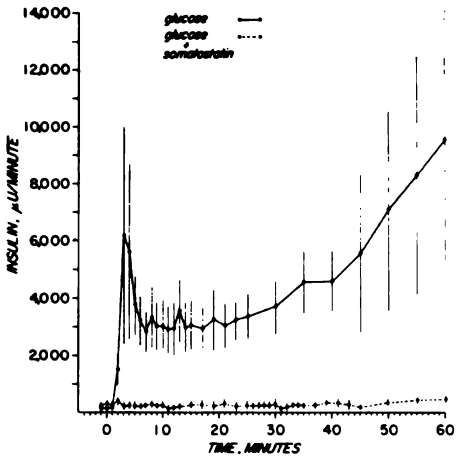

FIG. 1. Insulin secretion induced by 300 mg% glucose (solid line; $N = 6$) and inhibition by somatostatin (dashed line; $N = 5$). Somatostatin (100 ng/ml) was present from −15 to 25 min. Each point represents the mean ± SEM.

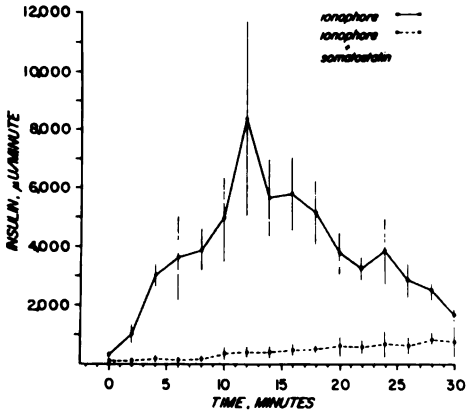

FIG. 2. Insulin secretion induced by 10 μM A23187 (solid line; $N = 6$) and inhibition by somatostatin (dashed line; $N = 4$). Somatostatin (100 ng/ml) was present from −15 to 30 min. Each point represents the mean ± SEM.

noted to suppress both phases ($P < 0.05$) of glucose-induced insulin secretion (Fig. 1, dashed line). Although the peptide was discontinued at minute 25, insulin secretion was suppressed throughout the 60-min perfusion, clearly indicating the potent inhibitory effect of the compound. Insulin secretion elicited by ionophore A23187 was also abolished ($P < 0.05$) in the presence of somatostatin with the secretory rate never rising above 800 μU/min (Fig. 2, dashed line). These results are in sharp contrast to the paradoxical increase in insulin secretion noted by Fujimoto and Ensinck (13) in rat islet cultures following administration of both somatostatin and A23187. This variance in data could possibly be due to differences in the concentrations of calcium and ionophore A23187 employed in the two studies (2.5 mEq/liter of Ca^{2+} and 10 μM A23187 compared to 1.8 mEq/liter of Ca^{2+} and 19.1 μM[2] ionophore for Fujimoto and Ensinck), or might be attributable to either differences in the species of β-cells tested, or to differences in the preparations used in the two investigations.

An increase in intracellular calcium in response to an appropriate stimulus is believed to be a necessary prerequisite for the initiation of insulin release (14). Recent reports have indicated that the inhibitory effect of somatostatin on glucose-induced secretion can be reversed by supraphysiological quantities of calcium, suggesting that somatostatin may act by inactivation of some calcium dependent event in the secretory process (8, 9). The insulinotropic action of ionophore A23187 in the perfused dog pancreas can be theorized to be due to the ability of this compound to elevate intracellular calcium, possibly by transporting the ion from the extracellular fluid. In support of this theory, we have previously demonstrated that ionophore-induced insulin secretion in the dog pancreas preparation is dependent upon an adequate perfusate calcium concentration (11). While conclusive data regarding somatostatin's mechanism of action is not currently available, we feel the present findings support the concept that somatostatin may act by uncoupling some calcium-related event critical to the insulin secretory process.

Summary. Cyclic somatostatin, at a concentration which inhibited both phases of glucose-induced insulin release, completely suppressed monophasic insulin secretion elicited by ionophore A23187. The ability of A23187 to initiate insulin secretion is believed to be highly dependent upon the drug's capacity to influence calcium transport. Thus, the present findings suggest that

[2] Calculated from the concentration of 10 μg/ml reported by Fujimoto and Ensinck.

the negative influence of somatostatin on the insulin secretory process may be exerted on some calcium-related event critical to the release mechanism.

We are grateful for the technical assistance of Mr. Ken Milburn and the secretarial aid of Mrs. Ruth Garrett and Miss Mitzi Spence.

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Received September 27, 1976. P.S.E.B.M. 1977, Vol 154.

INGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 201-205 (1977)

ıbilization of Initial Attachment Site Activity for Avian Tumor Viruses with Lithium Diiodosalicylate (39637)[1]

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troduction. Since membrane constitu- are frequently denatured during isola- preservation of their biologic activity solubilization provides a unique oppor- y to study cell surface components *in* . This report describes the solubiliza- of a cell surface attachment site for ı tumor viruses (ATV) from chicken yo fibroblast (CEF) plasma mem- es.

tachment to the cell membrane by a must be the first event in cellular infec- The Rous sarcoma virus genome /), with its Rous-associated virus enve- RSV(RAV-1) is the model we have to investigate the early phases of ATV- interactions. Initial attachment is de- ent, in the absence of polycations, intact viral surface glycoproteins (1). actions of the viral envelope and cell ce also determine the host range and ference patterns of the individual ATV roups (2). Cellular resistance to each subgroup is controlled by autosomal s (3), and susceptibility is dominant. ken embryo fibroblasts resistant to a cular ATV subgroup are still able to rb that virus (4); resistance is depend- ıpon later surface events which may spond to viral penetration (2). In order ıin insight into the cell surface events h influence the success of infection and mine some aspects of membrane struc- methods were sought which would ilize the initial attachment site for , while preserving its activity. Marchesi and Andrews (5) have reported that lithium diiodosalicylate would solubilize the princi- pal glycoproteins of the human erythrocyte and preserve their influenza-binding activ- ity. This compound was therefore utilized to solubilize attachment site activity for ATV from CEF plasma membranes.

Methods and materials. Reagents. Ribo- nuclease-free sucrose was obtained from Schwartz-Mann, Orangeburg, N.Y. All su- crose solutions (w/w) were prepared in TEN buffer (0.01 *M* Tris, 0.1 *M* $NaCl_2$, and 0.001 *M* EDTA, pH 7.4) and sterilized by filtration. Sodium [^{3}H]borohydride (490 mCi/mmole) was purchased from Amer- sham-Searle; [5-^{3}H]uridine (5 Ci/mmole) and [^{3}H]glucosamine hydrochloride (5–15 Ci/mmole) were from New England Nu- clear, Boston, Mass. Lithium 3,5-diiodosa- licylate (LIS) was obtained from Eastman Organic Chemicals, Rochester, N.Y. or syn- thesized according to Marchesi and An- drews (5); each preparation of LIS ap- peared equally active.

Cell culture and media. Tissue culture me- dia and calf sera were purchased from Grand Island Biological Company, Grand Island, N.Y. Primary chicken embryo fibro- blast cultures were prepared as previously described (1, 6) from 9-day-old gs- chick embryos obtained from SPAFAS, Inc., Rockford, Ill. Cells were passed 3 to 5 days after initial seeding and grown to confluence either for use in binding assays or for prepa- ration of plasma membrane fractions. Sec- ondary cells more than 7-days-old were not used. In selected experiments, secondary CEFs were treated sequentially with pyri- doxal phosphate (Sigma) and sodium [^{3}H]borohydride according to Rifkin *et al.* (7) or grown in the presence of 50 μCi/ml of [^{3}H]glucosamine for 48 hr before collection.

Virus preparation and purification. Bryan strain Rous sarcoma virus and Rous-associ- ated viruses RAV-1 and RAV-2 were used

ıpported by Public Health Service Grant CA and grants from the Graduate School of the rsity of Minnesota, the Minnesota Medical Foun- , the Minnesota Branch of the American Cancer y, and the Leukemia Task Force.

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ISSN 0037-9

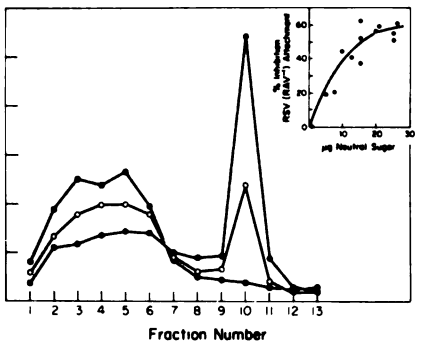
to prepare pseudotype RSV(RAV-1) and RSV(RAV-2) as described elsewhere (1). Supernatant fluid from infected cultures was replaced with media containing 20 μCi/ml of [^{3}H]uridine and harvested at 12-hr intervals. The virus-containing supernatant fluid was centrifuged to remove cell debris and concentrated by centrifugation over a discontinuous 20/60% sucrose gradient as previously described (8). Focus-forming units (FFU) were measured by established procedures (6).

Binding assay. Viral attachment to CEF plasma membranes were measured as reported elsewhere (1). Briefly, 200 μg (protein) of CEF plasma membrane was incubated with labeled ATV in TEN buffer at 4° for 25 min. These samples were then adjusted to 45% sucrose, layered upon a 60% sucrose cushion, and overlaid with 40 and 10% sucrose. This mixture was centrifuged at 90,000*g* for 20 min. Fractions were collected from the bottom, and each tube was assayed for acid precipitable counts per minute. CEF membranes and membrane-bound virus migrate to the 10/40% interface, while unbound virus remains with the 45% sucrose. This procedure is highly reproducible. Binding is dependent upon virus concentration, membrane protein concentration, and the presence of intact viral glycoproteins (1). The soluble attachment site material was assayed by incubating the labeled virus in the presence of fractions extracted (see below) from CEF membranes for 30 min at 4°, then adding intact CEF plasma membranes, and measuring attachment as above. Activity was quantified by the reduction in the amount of labeled virus bound after incubation with the LIS solubilized attachment site, compared to the amount bound after incubation in buffer but in the absence of the LIS solubilized site.

Solubilization of attachment site activity with LIS. CEF plasma membranes were prepared by a modification (1) of the procedure of Boone *et al.* (9). Approximately 5–10 mg of CEF plasma membranes (10- to 20-fold enriched for plasma membrane markers) was suspended in 0.3 *M* buffered LIS (0.05 *M* Tris, 0.1 *M* NaCl, pH 7.4). After being stirred at room temperature for 15 min, the supernatant fluid from this mixture was extracted with phenol, dialyzed, lyophilized, and washed with ethanol as described by Marchesi and Andrews (5). The pellet from the ethanol wash was dialyzed against TEN buffer, and particulate material was removed by centrifugation at 10,000*g* for 15 min. This material was then assayed for the capacity to inhibit virus attachment to plasma membranes. For selected studies, membranes were prepared from labeled CEFs or human erythrocyte ghosts and extracted as above.

Analytical procedures. The method of Lowry *et al.* (10) was used to assay protein with bovine serum albumin as standard. Neutral sugar was assayed according to Dubois *et al.* (11) with galactose as standard, and sialic acid was quantified according to Warren (12).

Results. Glycophorin (5) solubilized from human erythrocytes with LIS will bind influenza virus *in vitro*. Accordingly, we examined an LIS extract of CEF plasma membranes to determine whether this extract would interact with virus *in vitro* to impair subsequent virus binding by CEF plasma membranes. RSV(RAV-1) was incubated either with CEF LIS extract or buffer and then exposed to CEF membranes to assay viral attachment as in Methods and Materials (Fig. 1). Attachment was consistently reduced by the chick-cell LIS extract (Fig. 1). A representative study (Fig. 1) shows a 55% reduction in RSV(RAV-1) binding after incubation with 19 μg of CEF LIS extract. Attachment of RSV(RAV-2) (not shown) was inhibited to a similar degree by the LIS extract. Since the number of virus-binding sites is not known, conditions are regulated so that attachment is linear with respect to both membrane concentration and time. A general relationship between the percentage of depression of virus binding and the amount of LIS extract used can be seen (inset to Fig. 1). Inhibition appears linear to 10 μg (neutral sugar), and, then, the blocking efficiency per microgram of extract decreases. Activity varied with individual extracts, and the percentage of blocking per microgram of neutral sugar ranged from 2 to 5%. In several separate studies, LIS extracts of plasma membranes obtained from between 2 and 6 $\times$ 10^7 CEFs would



1. Inhibition of RSV(RAV-1) binding to CEF nes. Labeled RSV(RAV-1) (5 × 10^5 FFU, nately 1700 cpm) was incubated either with 19 ıral sugar) of CEF LIS extract for 30 min at 4°)), or in TEN buffer (⊙——⊙). CEF plasma ne (200 μg of protein) was then added to each, incubation continued for an additional 30 min assess the amount of attachment, RSV(RAV-—●) was also incubated in TEN buffer for the ne and then placed on the gradient without nes. Virus binding to the membranes was as- discontinuous sucrose gradient centrifugation thods and Materials. Inset: RSV(RAV-1) was d with increasing concentrations of CEF LIS or 30 min at 4°. Inhibition of virus binding of sma membranes was measured by comparing ing to that of RSV(RAV-1) incubated in TEN The curve is a computer derived least squares ghted to pass through zero.

the binding of 5 × 10^5 FFU of AV-1) by approximately 50%. The ual extracts had approximately equal ts of protein and neutral sugar, and anes from 3 × 10^6 CEFs yielded 1 μg (neutral sugar) of LIS extract. hibitory activity remained with the atant fluid after centrifugation at 0g for 30 min, and is recovered in the ıs phase after chloroform–methanol extraction. Preincubation of RSV-1) with LIS extract also reduced ctivity of the virus. RSV(RAV-1), 5 FFU, was incubated with 50 μg of tract or in TEN buffer at 4° for 60 ıd then titered in a standard FFU) determine infectivity. After 1 week, al number of foci present at two dilu- 10^{-3}, 10^{-5}) were counted; at both ıs, there was a 50% reduction in the r of foci with the LIS extract-treated ompared to virus incubated in TEN alone.

Evidence for a direct interaction between unlabeled RSV(RAV-1) and a LIS extract of CEF membranes is shown in Fig. 2; CEFs were labeled either on their surface with sodium [^{3}H]borohydride (7) or endogenously by growth in [^{3}H]glucosamine-containing media. Membranes prepared from each of these labeled cell preparations were then extracted with LIS as in Methods and Materials. The labeled LIS extracts were then incubated with unlabeled RSV(RAV-1) or with media, and these mixtures (labeled LIS extract ± RSV(RAV-1) were sedimented in discontinuous sucrose gradients. A peak of radioactivity (1–5% input counts) migrates with the RSV(RAV-1) (arrow), and much less radioactivity migrates to this region when sedimented after incubation in the absence of this virus. Nonspecific trapping of counts was eliminated since sedi-

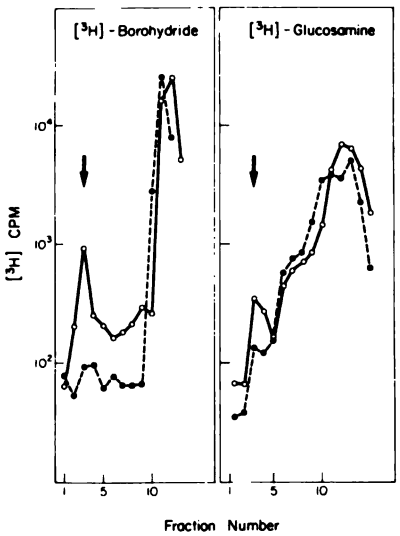


FIG. 2. Direct attachment of labeled LIS extract to RSV(RAV-1). CEF were labeled by sequential exposure to pyridoxal phosphate and sodium [^{3}H]-borohydride (7) (left) or by growth in media containing 50 μCi/ml of [^{3}H]glucosamine (right). LIS extracts of the plasma membranes from these cells were mixed with unlabeled RSV(RAV-1) (○——○) 1 × 10^8 FFU or media (●——●). These mixtures were incubated for 1 hour at 4° and sedimented on a discontinuous 20/60% sucrose gradient at 100,000*g* for 2.5 h; the gradient was collected from below, and the ethanol precipitable radioactivity in each fraction was measured. The migration of virus alone is indicated by the arrow. The labeled material migrating above fraction 9 represents material which failed to enter the gradient.

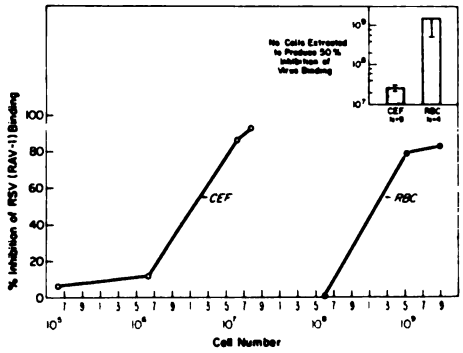


FIG. 3. Comparison of activity extracted from CEF membrane and human erythrocyte ghosts. CEF plasma membranes and human erythrocyte ghosts were extracted with LIS as in Methods and Materials. RSV(RAV-1) (5×10^5 FFU, approximately 1500 cpm) was incubated with these extracts or in TEN buffer for 30 min at 4°. CEF membrane (protein), 200 μg, was added and the incubation continued for 30 min at 4°. Binding of the virus to membranes was assayed by sucrose gradient sedimentation as described in Methods and Materials. The percentage of inhibition of RSV(RAV-1) binding is plotted against the number of cells required to produce the amount of extract giving that degree of inhibition. Inset: The number of cells to produce 50% inhibition of RSV(RAV-1) binding to CEF membranes. The number of separate studies and SEM is shown.

mentation of the labeled LIS extract with polio virus (3×10^6 $TCID_{50}$) did not reveal a similar peak at the 20/60% sucrose interface.

Glycophorin (5) prepared by LIS extraction of human erythrocyte ghosts was compared to a CEF LIS extract to determine the relative capacity of each to inhibit ATV attachment to CEF membranes. As illustrated in Fig. 3, glycophorin did not antagonize RSV(RAV-1) attachment as well as LIS extracts of CEF. Membranes from approximately 200 erythrocytes must be extracted to produce the activity of a single CEF. Other studies (not shown) indicate that labeled glycophorin did not bind directly to RSV(RAV-1).

Discussion. Previous studies [(1); Moldow, C. F., manuscript submitted for publication] indicated that virus binding to CEF plasma membranes is a sensitive measure of the initial attachment of ATV to the CEF surface. The data presented show that soluble LIS extracts of CEF plasma membrane reduce the attachment of at least two RSV pseudotypes to fresh CEF plasma membranes. RSV(RAV-1) incubated with the LIS extract is also less effective in transforming CEF than control virus, and evidence is presented which indicates that constituents of the LIS extract may bind directly to RSV(RAV-1) *in vitro*. We infer that some component of the LIS extract is binding to the ATV surface glycoproteins, preventing subsequent binding of the virus to its membrane attachment site. Since the viral RNA remains resistant to RNase (Moldow, C. F., unpublished observation) and infectious virus is still present after incubation with the CEF LIS extract (albeit at reduced levels), virus disruption by the LIS extract appears an unlikely alternate explanation.

Human erythrocyte membranes bind ATV poorly (1), yet, an LIS extract of these membranes interferes with ATV attachment to CEF membranes. Although precise quantitation is not possible, it appears that many more erythrocytes must be extracted to produce activity equivalent to that present on CEF. This difference is quantitatively similar to that reported for attachment site activity solubilized by trypsinization of CEF (Moldow, C. F., manuscript submitted for publication). Direct binding of LIS extract from labeled ghosts to ATV has not been demonstrated, and the erythrocyte extract may interfere with binding by an alternate mechanism. Quantitative and qualitative comparison of the activity present in various cells will ultimately require identification and purification of the attachment site activity solubilized from the CEF.

Summary. Extraction of CEF plasma membranes with LIS solubilizes membrane components which interact directly with ATV *in vitro*, antagonizing ATV binding to CEF plasma membranes and reducing the transforming capacity of these viruses. This activity is specific to CEF; approximately 200 human erythrocytes must be extracted with LIS to produce the activity equivalent to that extractable from a single CEF.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

Mechanism of Anti-Complementary Acti
Relevance in Endot

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Introduction. Corticosteroids protect animals from potentially lethal doses of endotoxin (1-6), but the mechanism for this protection remains as controversial as the mechanism for the toxicity of endotoxin itself. Recent evidence suggests that complement activation may be involved because: (a) Endotoxin added to serum *in vitro* activates complement predominantly through the alternative pathway (7); (b) patients in endotoxinemic shock manifest depletion of alternative pathway components (8); and (c) dogs (9) or rabbits (10) depleted of complement are protected from usually lethal doses of endotoxin. The possibility that corticosteroids may inhibit the deleterious effects of endotoxin by affecting the complement system is an attractive one. *In vitro*, corticosteroids depress the functional and immunochemical titers of the first four components of the complement cascade (11, 12), and, thereby, inhibit complement-mediated red cell lysis (11), immune adherence (11), and mast cell histamine release (13). Furthermore, since neutropenia commonly occurs with endotoxinemia, the recent observation that neutropenia is constantly associated with *in vivo* complement activation (14) lends further credence to the suggestion that complement activation may be critical in endotoxin toxicity. Thus, McCall *et al.* (14) have shown that *in vivo* activation of complement by intravascular injections into rabbits of cobra venom factor (CVF) or inulin, or, alternatively, infusion of plasma in which complement is preactivated by incubation with either agent, induces a profound transient neutropenia followed by neutrophilia. The neutropenia-inducing substance (NIS) was found to be a low molecular weight (<20,000 daltons) moiety formed when complement is activated by CVF or inulin.

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white rabbits, after which it was y chilled to 0° and centrifuged. Plasma moved, heparinized (1 U/ml), and l by 10% with either sterile normal or a corticosteroid dissolved in saline. luted plasma was incubated at 37° for l and then exposed to either polycellu- alysis membrane (20 cm²/ml) or inu- 2 mg/ml) for another 30 min at 37°. reparation was freed of inulin by cen- tion at 10,000 rpm, or of dialysis rane by manual removal, and then sed into the fermoral vein of the origi- nor animal over a 30-sec period. At s times thereafter, samples were from a femoral artery, and neutrophil were compared with those obtained ples taken less than 30 sec prior to n. In studies of *in vivo* complement ion, CVF, purified and assayed by ethod of Cochrane *et al.* (16), was l in a dose of 40 U/kg into the femo- n of 12 untreated control rabbits or orticosteroid-pretreated rabbits (two s at each dose). Blood samples were ed and analyzed as above. Hydrocor- 21-sodium succinate (Solu-Cortef), thylprednisolone 21-sodium succi- (Solu-Medrol), and dexamethasone dron) were diluted to appropriate trations in sterile normal saline and ed to pH 7.4 prior to incubation with l. Some animals were infused over 5- travenously with such prepared corti- oids prior to CVF infusion.

ults. Autologous plasma pre-exposed complement activator, cellophane ulin, not shown) rapidly induced re- ible transient neutropenia when rein- the degree of neutropenia was pro- nal to the quantity of plasma rein- (solid line, Fig. 1). The nadir of neu- ia developed within 3 min, and neu- ia occurred regularly in 10–30 min; ver, untreated plasma or plasma l of activatable complement by pre- g (56°) or hydrazine treatment lacked ctivity when reinfused (not shown). ubation of plasma with corticoste- rior to polycellulose membrane expo- hibits the neutropenia and does so in -related fashion; that is, the greater ount of activated plasma injected, the greater the level of corticosteroid needed for inhibition (interrupted lines, Fig. 1). Moreover, the concentrations of corticoste- roid required for inhibition are in the same range as those previously reported as in- hibiting complement-mediated red cell lysis (11), immune adherence (11), and mast cell histamine release (12) *in vitro*; as with other corticosteroid effects, Solu-Medrol is more potent than Solu-Cortef. Infusion of cortico- steroids alone induced minimal neutrophilia resembling the upper two lines of Fig. 1.

In marked contrast, if corticosteroids are incubated with plasma after complement has already been activated by incubation with cellophane (or inulin, not shown), inhi- bition of neutropenia does not occur (Table I). Analogously, if animals are treated di- rectly with corticosteroids (5–250 mg/kg of Solu-Cortef of Solu-Medrol) before infusion of complement-activated plasma, they de- velop neutropenia identical to that noted in untreated animals (Table I). These results demonstrate that corticosteroids inhibit the fluid phase formation of NIS, but, once this factor is formed, they have no effect on its action *in vivo*. Although only data utilizing cellophane-activated plasma are shown, identical results were obtained with infu- sions of plasma exposed to inulin.

Activation of complement *in vivo* by CVF infusion is also associated with neutropenia

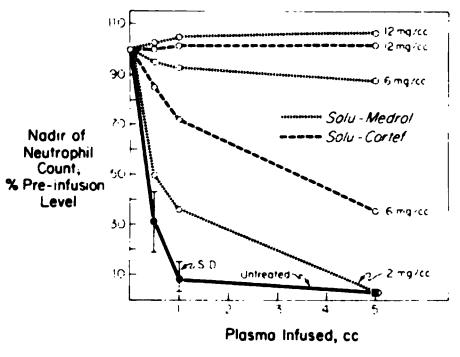

FIG. 1. Inhibition of C-induced neutropenia by corticosteroids *in vitro*. Infusion of increasing amounts of autologous plasma in which complement compo- nents had been activated by preincubation with di- alysis-coil cellophane produces progressively severe neutropenia in rabbits (solid line). If corticosteroids are added prior to cellophane exposure, a dose-related inhibition of the neutropenia effect results (interrupted lines).

TABLE I. LACK OF EFFECT ON NEUTROPENIA WHEN CORTICOSTEROIDS ARE USED AFTER COMPLEMENT ACTIVATION.

Infused plasma (ml)	Nadir of neutropenia (percentage of baseline) when		
	Untreated (N = 8)	Steroid added after C activation[a]	Steroid treated[b]
0.75	31.5 ± 5.6[c]	27	24.1 ± 9.3
1.00	8.4 ± 2.2	3.8 ± .2	6.2 ± 1.0
5.00	3.3 ± 0.8	2.0	3.9 ± 3.8

[a] Solu-Medrol (12 mg/ml) added to plasma after its treatment with dialysis membrane. Five animals were studied at the 1.00-ml dose and two animals each at the 0.75- and 5.00-ml doses of plasma.

[b] Solu-Medrol (60 mg/kg) infused with rabbit 10 min prior to complement-activated plasma infusion. Five animals were studied in each group.

[c] SE.

which lasts 30–60 min (solid line, Fig. 2). Neutropenia is inhibited by pretreating animals with Solu-Medrol in a dose-related manner (interrupted lines, Fig. 2); Solu-Cortef is similarly and appropriately effective. In striking contrast, Decadron is less effective, relative to its usual 20–30 and 5–8 times greater relative potency compared to Solu-Cortef and Solu-Medrol. Thus, minimally inhibitory doses of Solu-Cortef and Solu-Medrol given 10 min prior to CVF are 100 and 20 mg/kg, respectively; the expected corresponding dose of Decadron should be approximately 3.75 mg; instead, at least 15 mg/kg was required to inhibit neutropenia (not shown).

When corticosteroids are given at increasingly remote times before CVF injection, their effects are progressively lessened. For instance, a well-marked inhibitory effect of a given dose of Solu-Cortef administered 10 min before CVF becomes less effective if administered 30 min before, and is totally without effect if administered 90 min before the complement activator (Fig. 3). Moreover, if the dose of infused CVF is doubled (to 80 U/kg) a comparable increase in injected corticosteroid is required to significantly inhibit the induced neutropenia (not shown).

Discussion. These results allow two conclusions. (a) Corticosteroid inhibition of NIS formation appears to be reversible and related to the concentration of circulating corticosteroid and to the strength of complement activator injected. (b) Decadron is relatively impotent in preventing NIS generation; this relative impotence has also been noted in recent studies which compared the ability of various corticosteroids to inhibit neutrophil accumulation in inflammatory exudates (17), an accumulation which others have demonstrated to be dependent on complement activation (18).

The results of these *in vivo* experiments are also strikingly similar to those noted by others when corticosteroids were used to protect animals form endotoxin. Thus, high steroid doses are protective, and lower

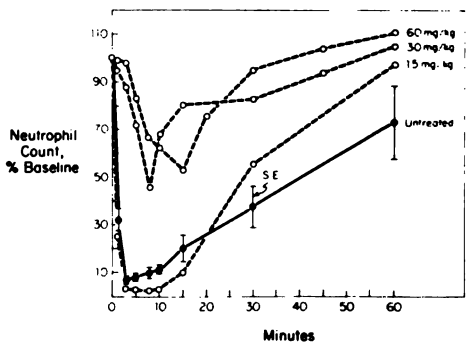

FIG. 2. Dose-related inhibition of CVF-induced neutropenia by corticosteroids *in vivo*. Rapid neutropenia occurs in rabbits in which complement is activated by administration, at 0 time, of purified cobra venom factor (solid line); an effect inhibited by high, but not low, doses of Solu-Medrol given 10 min before the CVF (interrupted lines). Solu-Cortef, but not Decadron, was also appropriately inhibitory (not shown).

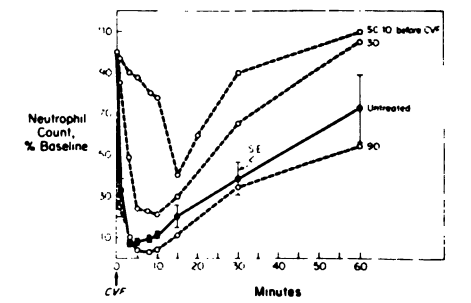

FIG. 3. Diminished activity of Solu-Cortef (SC) when administered at intervals remote from CVF. Although the usual neutropenia induced by CVF injection into rabbits (solid line) is significantly ameliorated by Solu-Cortef (150 mg/kg) given 10 min before CVF (top line), its inhibitory effect is progressively blunted when given at more remote times.

; are much less so (1, 5, 6). When 2700 were given intraperitoneally simulta- s lethal doses of endotoxin and cortico- ids, the doses of Solu-Cortef, Solu- ol, and Decadron needed to halve ality were 146, 70.9, and 18.6 mg, re- ively (1); that is, Decadron was rela- impotent. Furthermore, corticoste- given 0.5–1 hr prior to endotoxin were rotective than when given simultane- (5, 6), and, if given 2 hr prior, they not protective at all (6). As with the studies in the present report, if the nt of infused endotoxin was increased, rotection afforded by a given dose of osteroid was offset (5, 6). Taken to- r, these data suggest that corticoste- protection in endotoxinemia is also a sible one related to the dose of cortico- id and the amount of endotoxin in- l, and that Decadron is relatively ineff- e.

m these similarities, we suggest that lement-associated generation of NIS be involved in endotoxin shock. Fur- ore, our results indicate that, once is formed, corticosteroids are ineffec- n altering its effects. However, inhibi- f the further generation of this poten- deleterious substance is possible, but with very high concentrations of partic- two of these drugs, Solu-Cortef and Medrol; Decadron is less useful. In this d, it is of interest that previous studies e efficacy of corticosteroids in amelio- human endotoxin shock have been icting. If NIS generation is indeed criti- endotoxin shock, most of these studies l not have used doses of corticosteroids ient to block such generation. From esults presented, we suggest that very doses (perhaps as high as 30 mg/kg of Medrol or 150 mg/kg of Solu-Cortef) requent administration of these agents prove beneficial in human endotoxin , and studies employing such regimens d be underaken.

nmary. The effects of several cortico- ds on the sudden neutropenia which s in animals exposed to activated com- nt components were studied. Solu- ol and Solu-Cortef in high concentra- tions inhibited such neutropenia but only if present before or during the period of complement activation; Decadron was much less efficient. The observed dose and temporal relationships are strikingly similar to those noted in previous studies of corticosteroid protection from endotoxin shock, suggesting that complement-mediated alterations in neutrophils may be critical in this entity.

This work was supported in part by grants from the National Institutes of Health, AM 15730, CA 15627, and HL 19725.

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Received July 20, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 210-214 (1977)

Effects of Fasting on Insulin and Glucagon Secretion by Isolated Rat Islets of Langerhans (39639)

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Introduction. Fasting results in changes of hormonal balance which favor the provision of substrates necessary for the continued production of energy. An important hormone in this regard is glucagon. In pharmacological amounts, this hormone stimulates glycogenolysis and gluconeogenesis in the liver (1, 2) and the release of fatty acids from adipose tissue (3, 4). Glucagon concentrations in the blood have been shown by some investigators to increase markedly during periods of glucose deprivation (5, 6), but others have shown little or no change (7, 8, 9). Interpretation of such conflicting observations made *in vivo* is complicated by a number of factors. Thus, the antisera used for radioimmunoassay of glucagon have not always been specific for pancreatic glucagon; they can cross-react with other substances present in plasma (10, 11). In addition, it is known that fasting can cause other hormonal changes which directly affect metabolism at the tissue level and which could directly or indirectly influence glucagon secretion from the pancreas *in vivo*. In order to determine whether fasting has any direct effect upon pancreatic endocrine function, glucagon and insulin secretion were measured in isolated islets of Langerhans obtained from rats killed in the fully fed state or after fasting for periods of 24 to 72 hr.

Materials and methods. Male Sprague-Dawley rats (250-300 g) had free access to water and were fed *ad libitum* on Purina rat chow except during periods of fasting. All experiments were performed between 0900 and 1100 hr. Animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg body weight). After laparotomy, blood was collected by cardiac puncture into a heparinized syringe and chilled in tubes containing Trasylol (F.B.A. Pharmaceuticals, N. Y., 200 U/ml of blood). The blood was centrifuged, and plasma separated within 5 min of collection; the plasma was stored at −20° until assayed for glucagon. Glucose was determined in each sample of blood before addition of Trasylol using an Ames reflectance meter (Ames, Elkhart, Ind.).

Islets were isolated by the collagenase digestion procedure of Lacy and Kostianovsky (12). Briefly, this procedure involves cannulation of the common bile duct and injection of approximately 20 ml of buffered Hanks' solution to disrupt the acinar tissues of the pancreas. The pancreas is removed, cleaned of all connective and extraneous tissue, chopped into small pieces, and exposed to collagenase (6-10 mg/pancreas; Type IV, Worthington Biochemicals, N. J.) during rapid hand shaking at 37° for 7-10 min. Enzymatic digestion of the pancreas is terminated by 4 × 10-ml buffer washes and centrifugation steps. After a final centrifugation, the pellet of pancreas and islet tissue is poured into siliconized glass petri dishes, and the islets are removed with a small glass loop. Approximately 150 islets were transferred to a Swinnex Millipore chamber (13 mm; Millipore, Bedford, Mass.) containing a 5-μm pore size membrane. The chamber containing islets was submerged in a water bath maintained at 37° and perifused with Krebs Ringer bicarbonate buffer supplemented with bovine plasma albumin (0.2%; Fraction V, Sigma Chemical Company, St. Louis, Mo.). The perifusion medium was maintained at pH 7.4 with a 95% O_2:5% CO_2 gas mixture.

Experimental protocol. During each experiment, two chambers were perifused simultaneously at a flow rate of 1 ml/min. One chamber contained islets from a fed rat and the other chamber islets from a fasted rat. An equilibration period of 15-20 min preceded the beginning of perifusate sample collection. Effluent samples (0.1 ml), col-

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ISSN 0037-9727

in the presence of 500 U of Trasylol, nmediately frozen and stored at −20° ssayed for insulin and glucagon. Islets stimulated with arginine (10 m*M*; Chemical Company, St. Louis, Mo.) a combination of arginine (10 m*M*) ucose (16.7 m*M*).

oimmunoassay procedures. Insulin termined by the method of Makulu *et*) using alcohol precipitation for the tion of antibody bound from free hor- Glucagon was determined using an um specific for pancreatic glucagon double antibody technique slightly ed (14) from the method of Pagliara 15). Porcine insulin and glucagon (Eli Company, Indianapolis, Ind.) were s standards and for labeling with io- 25.

analysis. Students *t*-test was used for cal analysis of data. A *P* value of 0.05 was considered to be significant.

lts. Blood glucose and plasma gluca- Table I shows blood glucose and glucagon levels for fed animals and s fasted for periods of 24, 48, and 72 lucose concentrations were signifi- lower for all fasting animals when red with fed controls ($P < 0.001$). gon concentrations were not altered ing.

fusion studies. As shown in Fig. 1–3, perifused in low glucose media (1.67 nd stimulated with arginine (10 m*M*) 0 min, exhibited biphasic secretion of on. Fasting for 24, 48, or 72 hr had r no effect on either the first or sec- ase of secretion. Increasing the con- ion of glucose in the perifusion media 7 m*M* after 30 min resulted in a de- of glucagon secretion by islets from ed and fasted animals. Addition of e (10 m*M*) to islets perifused in low glucose (1.67 m*M*) media did not affect basal insulin secretion rates. Increasing the glucose concentration to 16.7 m*M* then stimulated biphasic insulin secretion by is-

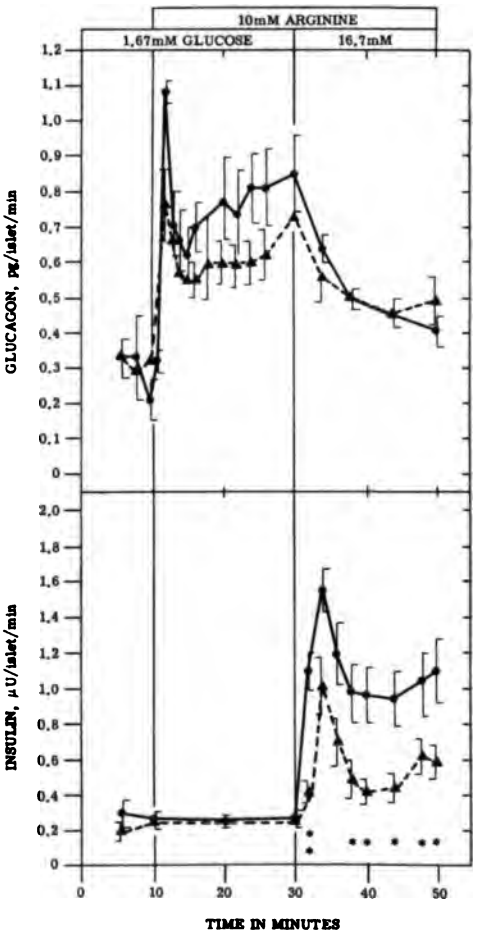

FIG. 1. Upper panel shows glucagon secretion rates by islets prepared from fed (●——●) and 24-hr fasted (▲-----▲) rats. Lower panel shows insulin secretion rates for the same islets. Each point represents the mean ± SEM for six observatons. (*) Represents $P < 0.05$, and (**) represents $P < 0.005$.

BLE I. BLOOD GLUCOSE AND PLASMA GLUCAGON CONCENTRATIONS FOR FED CONTROL AND FASTED ANIMALS[a]

of fast (hr)	Glucose (mg/100 ml)		Glucagon (pg/ml)	
	Fed control	Fasted	Fed control	Fasted
24	166 ± 5$_a$ (6)	95 ± 4$_b$ (6)	188 ± 24$_g$ (6)	204 ± 28$_h$ (6)
48	145 ± 4$_c$ (22)	84 ± 4$_d$ (20)	167 ± 13$_i$ (22)	146 ± 12$_j$ (19)
72	156 ± 4$_e$ (16)	105 ± 5$_f$ (14)	142 ± 10$_k$ (14)	137 ± 11$_l$ (13)

istical analysis: $P < 0.001$ for a versus b, c, versus d, and e versus f. There were no significant differences g and h, i and j, or k and l. Numbers of observations are shown in parentheses.

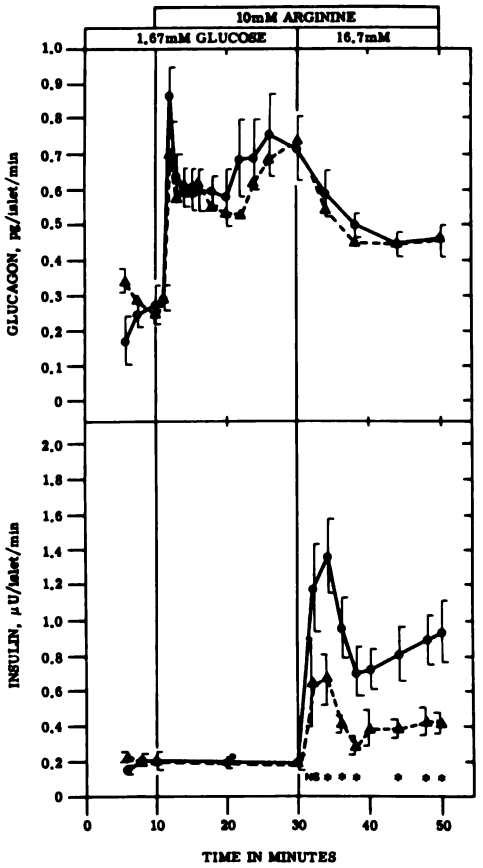

FIG. 2. Upper panel shows glucagon secretion rates by islets prepared from fed (●——●) and 48-hr fasted (▲-----▲) rats. Lower panel shows insulin secretion rates for the same islets. Each point represents the mean ± SEM for six observations. (*) Represents $P < 0.05$.

lets from both fed and fasted animals. Fasting was seen to cause significant reductions in both phases of insulin secretion. The onset of insulin secretion in response to glucose was not altered.

Discussion. Results from these studies indicate that, while fasting (24–72 hr) caused a significant decrease in blood glucose concentration, plasma glucagon concentration was not significantly affected. Furthermore, when stimulated with arginine islets, from fed and fasting animals demonstrated similar glucagon secretion rates. Buchanan *et al.* (8), using 3-day fasted rats, obtained similar results. However, in their studies, flask incubated islets were used, a system which does not permit either detection of subtle changes in secretion rate or analysis of the dynamics of glucagon secretion. In experiments using fed and 5-day fasted dogs, Buckman *et al.* (9) were unable to show any differences between plasma glucagon concentrations either in the basal state or during an alanine tolerance test.

Contrary to our findings are those of Aguilar-Parada *et al.* (7) who showed slightly elevated plasma glucagon concentrations in 3- to 4-day fasted human subjects and marked elevations in glucagon secretion in response to arginine infusion. Other investigators (5, 6) have reported increases in plasma glucagon concentrations after fasting, in man. Also, experiments carried out in the rat (16) demonstrated elevated

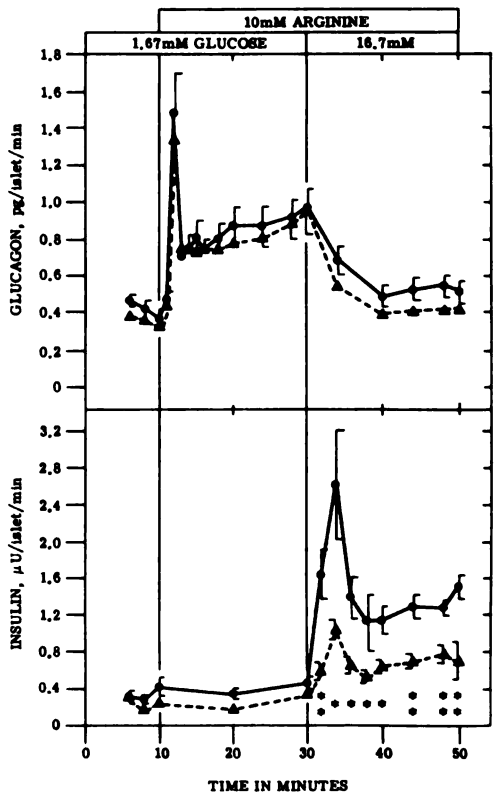

FIG. 3. Upper panel shows glucagon secretion rates by islets prepared from fed (●——●) and 72-hr fasted (▲-----▲) rats. Lower panel shows insulin secretion rates for the same islets. Each point represents the mean ± SEM for seven observations. (*) Represents $P < 0.05$, and (**) represents $P < 0.005$.

lucagon concentrations, and, in the (17), increased glucagon secretion islets from starved animals. The or differences between our findings e of others is not readily apparent, may be due to differences in experi- esign or animal species selected for

ard to insulin secretion, it is readily from our results that after 24 hr o 72 hr of fasting, insulin secretion re markedly reduced. Similar find- educed insulin secretion after fast- been reported by many investiga- -19). However, our results show, irst time, that fasting will suppress ses of insulin secretion, indicating a loss of sensitivity by the islets. Ma- *al.* (17) and Coddling *et al.* (18) gested such a mechanism on the an altered threshold concentration glucose stimulates insulin secre- other possible explanation for re- sulin secretion is decreased concen- f cyclic AMP in islet tissue during 0). Islet content of insulin does not o be an important factor since Ma- *al.* (17) and Buchanan *et al.* (8) wn little or no change in insulin of islets prepared from fed and 8–72 hr) rats.

gnificance of our results in explain- ole of glucagon on glucose homeo- difficult to assess. However, the re consistent with the thesis of Un- that, during periods of acute glu- rivation or prolonged fasting, it is nce between glucagon and insulin ntrols blood glucose concentration. *in vitro* have clearly shown the regulatory roles of these two hor- carbohydrate (22, 23) and fat me- (24). Thus, increases in plasma concentration may not be required sting, since the inhibitory effects of the glucogenic actions of glucagon educed at the lowered plasma insu- ntration associated with this state. , because of the many hormonal occurring during periods of glucose on, the exact mechanism for main- of blood glucose still remains to be d.

Summary. Rats fasted for periods of 24, 48, or 72 hr showed no changes in plasma glucagon concentration despite lowered blood glucose concentration. Islets isolated from both fed and fasted animals elicited biphasic glucagon and insulin secretion when stimulated with arginine and glucose, respectively. There were no differences in glucagon secretion rates between islets prepared from fed and fasted animals. Significant reductions in both the first and second phases of insulin secretion were observed in islets prepared from fasting animals.

The authors wish to thank Ms. Carol Grimme and Ms. D. Barr for typing the manuscript, and Dr. A. Pagliara for anti-glucagon serum. This work was made possible by Public Health Service Grant AM-16534.

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Received June 4, 1976. P.S.E.B.M. 1977, Vol. 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 215-218 (1977)

on of Lymphotoxin- and Lymphocyte-Mediated Cytolysis by High Molecular-Weight Dextran (39640)[1]

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>hotoxin (LT) is a cytotoxin released gen- or mitogen-stimulated lympho- *vitro* (1). The inhibition of lympho- :diated cytolysis by anti-LT serum well as other lines of evidence (1), that this lymphokine has a role in cyte-mediated cytotoxicity in tissue . Recently, Henney (3) and Ferluga lison (4) reported the protective ef- dextrans on lymphocyte-mediated ell destruction.

is study, we examined the effect of olecular-weight dextran (83,000) on toxin-induced cytolysis in tissue cul- d compared our findings with those d with dextran in cell-mediated cy- *n vitro*.

rials and assays. target cells. Mono- of mouse L fibroblasts, clone 929, for 24 hr in round-bottomed tubes, ed as targets. The tissue culture me- as RPMI 1640 with 5% fetal calf FCS).

photoxin. Human lymphotoxin was l from supernatants of phytohemag- (PHA)-stimulated human adenoi- phocytes as previously described (5). ivity of LT is expressed in cytotoxic CU) as formerly defined (5). Lym- n was dissolved in a buffer consisting *M* glycine–0.15 *M* NaCl, pH 9.5.

n cells. Sensitized spleen cells were d from BALB/c mice which had re- ntraperitoneal and, 3–4 weeks after- intrasplenic injections of living L erived from a C3H mouse as previ- escribed (6). Spleens were harvested ys later; RBCs were lysed in hypo- olution, and the spleen cells were resuspended in tissue culture medium. Control spleen cells were taken from noninjected BALB/c mice.

Dextran solutions. Dextran, average mol wt 83,000 (Sigma Chemical Co., St. Louis, Mo.), was dissolved in RPMI 1640 and dialyzed against this medium. After dialysis, the concentration of dextran was adjusted to the desired molarity by adding RPMI 1640. Then, the material was filtered through Millipore membranes (pore size, 0.22 μm), and FCS was added to yield a final concentration of 5% serum. Since nondialyzed dextran was found to be slightly cytotoxic, the dialysis was necessary.

Calculation of results. The effect of LT was assayed by the release of ^{51}Cr into the supernatant from L cells [prelabeled as described (7)], and radioactivity was measured in a gamma counter. For each determination, cultures were used in triplicate, and radioactivity was counted to a 1% error. The same technique, employing ^{51}Cr release, was used for assaying the effect of spleen cells on prelabeled target L cells.

The percentage of specific release (SR) of ^{51}Cr from prelabeled target cells was expressed as follows. $SR = (E - C)/T \times 100$, where E is radioactivity in supernatants of cultures receiving LT or sensitized spleen cells, C is radioactivity in supernatants of control cultures, and T is total releasable radioactivity). The percentage of inhibition of specific ^{51}Cr release was calculated relative to cultures without dextran.

Employing an electronic particle counter as described (5, 8), we also determined the numbers of lymphotoxin-treated target cells. Each determination was performed on five replicate cultures.

Results. Release of ^{51}Cr in LT-treated and control cultures as a function of time. The release of ^{51}Cr from LT-treated and control target cells was assessed as a function of time, using LT (700 CU) or control buffer.

orted by a grant from the National Institutes , U. S. Public Health Service: No. CA-07191-

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1977 by the Society for Experimental Biology and Medicine erved.

ISSN 0037-97

In this initial experiment, a markedly greater release of ^{51}Cr from LT-treated cells was observed as compared to that in control preparations (Fig. 1). From these data, we calculated the percentage of specific release, which indicated the percentage of release effected by lymphotoxin (Fig. 2).

Effect of dextran on LT-induced cytolysis as measured by ^{51}Cr release. Monolayers of ^{51}Cr-labeled target L cells (60,000 cells/ml) were incubated with 700 CU of LT. We conducted the experiments by two different methods: (i) by incubating the cultures with LT for 2 hr, removing the LT-containing medium, rinsing the preparations with Hanks' balanced salt solution (HBSS), and adding various concentrations of dextran in medium; or (ii) by simultaneously adding lymphotoxin and dextran to the medium. The final concentration of dextran was 0, 0.5, 1, or 1.5 m*M*, respectively. In both types of experiments, after the addition of lymphotoxin at 37°, the incubation period was 17 hr. Control cultures received equivalent amounts of buffer and dextran.

These experiments revealed a dose-related, dextran-induced inhibition of ^{51}Cr release (Fig. 3, Table I). Furthermore, we observed no difference, regardless of whether the cells were exposed first to LT and then to dextran, or both substances were added simultaneously. In one additional experiment (results not shown), inhibition of LT-induced target-cell lysis by dextran was confirmed using cell counting with a Coulter particle counter instead of ^{51}Cr release as a measure of cytolysis.

Effect of high molecular-weight dextran on target-cell lysis induced by sensitized spleen cells. Monolayers of ^{51}Cr-labeled target cells (60,000 cells/ml) were incubated with sensitized mouse spleen cells in a ratio of 1:20 for 2 hr at 37°. The supernatants containing nonattached spleen cells were removed, the monolayers were gently rinsed with HBSS, and various concentrations of dextran in medium were added. The final concentration of dextran was 0, 0.25, 0.5, 1, or 1.5 m*M*, respectively. Following the addition of the lymphocytes at 37°, the cultures were incubated for 17 hr. Control cultures received spleen cells from nonsensitized mice and equivalent amounts of dextran. Three experiments were performed.

Again, dose-dependent inhibition of ^{51}Cr release by dextran was observed in these

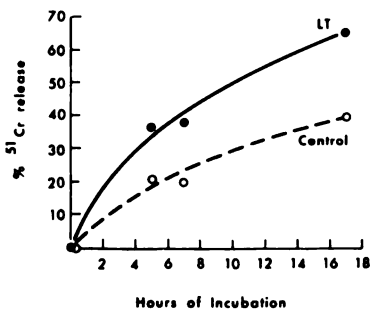

FIG. 1. Release of ^{51}Cr, as a function of time, from prelabeled target cells treated with LT (700 CU) or buffer.

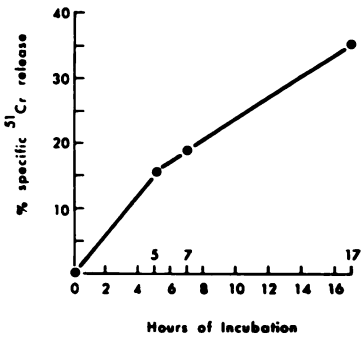

FIG. 2. Specific release of ^{51}Cr, as a function of time, from prelabeled target cells reflecting LT-induced injury.

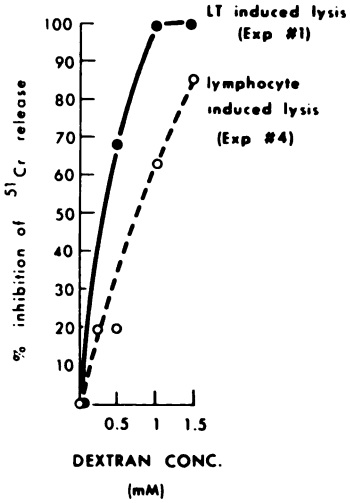

FIG. 3. Dextran-mediated inhibition of ^{51}Cr release induced by LT or sensitized spleen cells.

ABLE I. EFFECT OF DEXTRAN ON CYTOLYSIS INDUCED BY LT AND SENSITIZED SPLEEN CELLS.

	Percentage of inhibition of ^{51}Cr release[a]							
	Inhibition of LT-mediated lysis (%)				Inhibition of cell-mediated lysis (%)			
	Dextran concentration (m*M*)				Dextran concentration (m*M*)			
	0.5	1	1.5		0.25	0.5	1	1.5
:pt 1	68	100	100	Expt 4	20	20	64	85
:pt 2	11	15	60	Expt 5	22	46	61	60
:pt 3	25	38	100	Expt 6	–	32	25	56

ese experiments, the percentage of specific release of ^{51}Cr in the absence of dextran was 35–40.

:plicate experiments (Fig. 3, Table

ussion. In preliminary experiments, blished that ^{51}Cr release can be used id indicator of LT-mediated cell lysis the experimental conditions em- (Figs. 1 and 2). We had already :d previously that there was a close tion between the percentage of lysis rmined by cell count and the specific of ^{52}Cr from cytotoxically affected cells. The experiments were con- *in vitro* because only in such a system ssible to precisely define the macro- lar environment of the target cells quantitatively establish the interfer- ct of macromolecules on LT-induced s.

)bservations reported here clearly in- that LT-induced cytolysis can be in- by high molecular-weight dextran)) and that the degree of inhibition is :pendent. The reason for this inhibi- unknown, but four possible mecha- ıre suggested. (i) The polymer may :e with the binding of LT. (ii) Dex- ıy affect the cell membrane. (iii) In- ı may be attributable to the osmotic ıf the polymer. (iv) Inhibition may be the diffusion-limiting effects of dex-

iously, we noted the binding of LT to e target cells, with 50% of the maxi- ıding occurring within 2 min and pro- ş to full maximal binding in 1–2 hr though the rate of cytolysis is dose- ent, it becomes marked only several ıfter addition of the lymphokine un- e given experimental conditions.)re, we pretreated the target cells mphotoxin in Expts 1 and 2 for 2 hr to allow for uninterrupted maximal binding, removed the unbound LT, and then added the dextran to the target-cell cultures. Inhibition of lysis was observed under these circumstances just as in the case of the simultaneous addition of lymphotoxin and the polymer (Expt 3), intimating that dextran does not act by blocking the binding sites of LT and, thus, speaking strongly against the first mechanism considered.

It is also conceivable that the polymer in some way alters the function of the cell membrane. This hypothesis is difficult to test in view of the current incomplete knowledge of membrane structure and function as well as the various possible sites of action of the polymer on the cellular membrane. At this time, there is still conflicting opinion concerning the mechanism of intracellular fluid regulation. A primary role for the membrane, with pumps for different molecules, is a widely accepted premise (10), but regulation of intracellular fluid according to the state of hydration of macromolecules within the cell has also been postulated (11).

Another explanation which might serve is that high molecular-weight dextran dissolved within the tissue culture medium protects cells with LT-induced membrane injury by its osmotic effect. This theory is based on Henney's studies (3) of target-cell lysis by sensitized allogeneic spleen cells in the presence of dextrans of different molecular weights. He noted that small molecules were released at an early stage of cell damage, with subsequent release of macromolecules or markers bound to them. Moreover, he suggested that the enlargement of initial target-cell lesions by water influx, with the resultant release of cellular macromole-

cules, could be prevented by maintaining the injured cells in a medium containing high molecular-weight molecules. Such molecules, incapable of entering the cells, would prevent the influx of water by their osmotic effect.

Seeman, using erythrocytes, has observed that release of hemoglobin from red cells in a hypotonic environment can be reduced by the presence of macromolecules (12). Such an effect does not depend on the molecular weight of the macromolecule, but rather on its concentration by weight. He has suggested that, since macromolecules are highly hydrated, they exclude the solvent volume accessible to a second macromolecule (e.g., hemoglobin), thereby reducing the solubility and diffusability of the second molecule (13).

In the present study, dextran also inhibited target-cell lysis mediated by sensitized lymphocytes, confirming previous observations by other investigators (3, 4). Likewise, dextran interference with lymphocyte attachment is improbable, because the polymer was added after target-cell exposure to lymphocytes. Inhibition of lymphocyte- and LT-induced cytolysis by high molecular-weight dextran is consistent with the contention that both phenomena are mediated by the same final pathway.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

)F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 219-223 (1977)

ıce of Immunoreactive Luteinizing Hormone-Releasing Hormone in Ovine Cerebrospinal Fluid Collected from the Third Ventricle (39641)[1]

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ıntial quantities of luteinizing hor-
leasing hormone (LHRH) can be
d from the median eminence (1).
ıal brain areas have been implicated
the synthesis of LHRH or its stor-
use their cell bodies contained ma-
ıtochemically identified as LHRH
ɔecause tissue extracts contained
ıctivity (1). Among these, the cir-
ricular organs (3), including the
gan (4), are especially pertinent to
ɜnt paper because it has been hy-
ɜd (5) that the ventricular system
a route for transport of LHRH and
ɔophysiotropic factors. According to
ıthesis, LHRH might be secreted by
ıe circumventricular organs into the
pinal fluid (CSF), recovered from
by ependymal cells lining the floor
rd ventricle, and transported across
ian eminence (ME) to be released
ɔortal veins supplying the adenohy-
. The second and third aspects of
ɔthesis have already been proven
ıince LHRH can be recovered from
lood following its intraventricular
(6, 7). Furthermore, the intraven-
oute of LHRH administration (6,
ıdily causes pituitary release of lu-
hormone (LH).

ogenous LHRH is transported by
ricular system as hypothesized (5),
ous LHRH should be recoverable
rd ventricle CSF. Unfortunately,
ł results on this important point are
ɜtory. Joseph *et al.* (10) reported
', collected after death from ovari-
d rats, contained large quantities
g/ml) of immunoreactive LHRH.
ɜly, Cramer and Barraclough (11)
t detect immunoreactive LHRH in artificial CSF perfused through the third ventricle of anesthetized female rats electrically stimulated to release LH. Technical differences in methods of CSF collection and of LHRH radioimmunoassay make difficult any resolution of the contradictory results from these two reports. Therefore, the present study was conducted with ovariectomized sheep, a larger experimental species and one in which substantial volumes of CSF could be collected repetitively from the third ventricle of conscious animals. Furthermore, LH release could be both inhibited and stimulated by estradiol administration in this preparation.

Materials and methods. Intraventricular cannulae made from 20-gauge stainless steel hypodermic needles were permanently implanted into the third ventricle of three adult ewes ovariectomized 3 months earlier. Stereotaxic procedures (Malven, unpublished) for locating the third ventricle were aided by dorsal-ventral radiographs obtained by the method of Tarttelin (12) with minor modifications. When the cannula tip was in the third ventricle, CSF readily flowed to the top of the cannula. Following recovery from surgery, a teflon cannula was introduced into the jugular vein for collection of blood plasma. During experimental trials, CSF (0.4 to 0.5 ml) and blood (2 to 4 ml) were collected simultaneously at 1or 2-hr intervals for periods of about 24 hr. In three such trials, 50 μg of estradiol-17β ($E_2\beta$) was injected intramuscularly 2 hr after the initiation of sample collection. There was one control trial in which no $E_2\beta$ was administered. Extra blood samples were sometimes collected at 10- or 20-min intervals for periods of 4 to 6 hr in order to analyze the pattern of episodic LH release. One additional trial was performed in which 50 μg of synthetic LHRH was rapidly injected through the jugular vein cannula.

ł paper No. 6350, Purdue University Agri-
ɜperiment Station, West Lafayette, Indiana

977 by the Society for Experimental Biology and Medicine
rved.

ISSN 0037-9727

Blood was collected at 2.5- to 10-min intervals from 90 min before to 150 min after LHRH injection. Third ventricle CSF was sampled at −90, −30, +5, +15, +30, +90, and +150 min relative to LHRH injection.

All samples of CSF were frozen on dry ice within 1 min after collection. Concurrently collected samples of blood were immediately centrifuged, and the separated plasma was frozen within 20 min. Additional blood samples, without a paired CSF sample, were kept at 4° for up to 6 hr prior to centrifugation. All samples of blood plasma and CSF were stored at −20° until immunoassayed for LH or LHRH.

In order to estimate potential losses of endogenous LHRH during storage and assay procedures, known quantities of synthetic LHRH were added to seven samples of freshly collected CSF. Synthetic LHRH (300 to 2700 pg) was also added to 2-ml aliquots of heparinized whole blood which were then incubated at 4° for 30 to 120 min prior to separation of plasma and storage at −20°. The pituitary stalk-median eminence region was dissected immediately after death in two of these ewes, weighed, and frozen for subsequent extraction and LHRH assay. The location of the cannula tip within the third ventricle of each ewe was verified by gross dissection of the ventral surface of the brain.

Radioimmunoassays (RIA). Concentrations of LH in blood plasma were quantified by procedures described previously (13). The antiserum developed and characterized by Kerdelhue *et al.* (14) was used to measure LHRH in CSF, blood plasma, and stalk-median eminence extracts. Synthetic LHRH (Beckman lot No. 1 337702) was used as standard, and the same material was radioiodinated (^{125}I) by the chloramine-T method and purified by gel filtration on Sephadex G-25. Radioactively labeled LHRH was added to the assay in a volume of 100 μl. The assay diluent was 0.1% gelatin in 0.01 *M* phosphate-buffered saline, pH 7.5, (gelatin-PBS), and it was added to each assay tube in variable volumes so that the total volume of diluent plus standard or diluent plus sample equaled 500 μl. Antiserum against LHRH (1:50,000) was added in a volume of 200 μl, yielding a final concentra-

TABLE I. CONCENTRATIONS OF LH IN PLASMA AND LHRH IN PLASMA AND CSF IN UNTREATED AND $E_2\beta$-TREATED EWES AND AFTER INTRAVENOUS LHRH INJECTION.

Hormone measured	Endocrine treatment of ewes				
	Untreated (n = 1[a])	$E_2\beta$ Treated (n = 3)		LHRH treated (n = 1)	
		Inhibitory periods (2–10 hr)	Stimulatory periods (12–20 hr)	Before LHRH	After LHRH
Plasma LH (ng/ml)	9.9 ± 0.8[b] (14)	1.5 ± 0.1[c] (31)	11.4 ± 1.1 (36)	1.4 ± 0.3[c] (10)	42.0 ± 4.6[c] (19)
CSF LHRH (pg/ml)	<100 (14)	<100 (20)	<100 (24)	<100 (3)	<100 (5)
Plasma LHRH (pg/ml)	66 ± 7 (14)	67 ± 9 (31)	43 ± 6[d] (36)	73 ± 4 (3)	251 ± 47[c] (9)

[a] n = Number of experimental trials.
[b] All means are presented ± SE with the number of samples in parentheses.
[c] Significantly different ($P < 0.01$) from average in untreated trials (Student's t-test).
[d] Significantly different ($P < 0.05$) from average in untreated trials (Student's t-test).

no LH surge took place although pulsatile LH release recurred between 12 and 20 hr after $E_2\beta$ injection. The failure of a sustained LH surge to occur in this one trial resulted in a mean plasma LH lower then expected, 12–20 hr after $E_2\beta$ (Table I).

Both Table I and Fig. 1 contain data from one trial in which blood plasma and CSF were collected before and after intravenous injection of 50 μg of synthetic LHRH. As expected, LHRH injection immediately increased plasma concentrations of immunoreactive LHRH with a maximum value of 420 pg/ml occurring at 2.5 and 5.0 min after injection. Plasma LHRH concentrations decreased rapidly thereafter, reflecting a fast clearance of the exogenous hormone from the blood plasma. Plasma LH also increased 2.5 min after injection, but, in contrast to LHRH levels, plasma LH continued to increase, reaching a maximum of 80 ng/ml at 70 min after LHRH injection. In both the present study and that of Jonas *et al.* (17), plasma LHRH returned to pretreatment levels prior to the maximal plasma LH concentration. Despite the large increase in plasma LHRH concentration following its injection, no immunoreactive LHRH could be detected in CSF samples (minimum detectable concentration = 100 pg/ml). These results and those of Cramer and Barraclough (11) in rats suggest that significant amounts of LHRH did not pass from blood into CSF.

The failure to find immunoreactive LHRH in natural CSF collected from the third ventricle cannot be attributed to the lack of a suitable RIA. The two SME extracts contained an average of 26 ng/ovine SME which was comparable to the 30 ng/ovine hypothalamus reported previously (14). Also, the collection and storage procedures for CSF did not appear to destroy immunoreactive LHRH. The recovery of exogenous LHRH, added to freshly collected CSF, averaged 142 ± 7%.

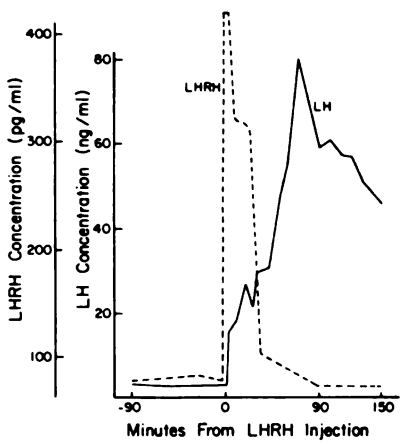

FIG. 1. Plasma concentrations of LH (solid line) and LHRH (dotted line) before and after intravenous injection of 50 μg of synthetic LHRH in one trial.

The present results with natural CSF confirm the observations of Cramer and Barraclough (11), using artificial CSF perfused through the third ventricle of anesthetized female rats. The present results contradict those in which relatively high levels of LHRH were recovered from CSF collected

from ovariectomized female rats (10) and from male rats (18) after death. Using a bioassay for LHRH rather than an immunoassay, Gradwell and Symington (19) also failed to find LHRH-like activity in bovine CSF collected from the spinal region.

In contrast to CSF, immunoreactive LHRH was detected in blood plasma but in highly variable concentrations. Recovery of exogenous LHRH, added to whole blood, averaged only 49%, but the rigorous extraction procedure probably contributed to this low average. LHRH concentrations in plasma samples from an untreated ovariectomized ewe averaged 66 pg/ml. (Table I). Plasma concentrations of LHRH did not change during inhibitory periods in $E_2\beta$ trials; however, during stimulatory time periods after $E_2\beta$ (12–20 hr), plasma LHRH tended to be reduced ($P < 0.05$). This inverse relationship between plasma LHRH and plasma LH concentrations might reflect a more efficient pituitary uptake of endogenous LHRH from portal vein blood during periods of $E_2\beta$-stimulated LH release. This interpretation of the present data contradicts the assumption of previous workers that concentrations of LHRH in the peripheral blood plasma of sheep might reflect the rate of release of endogenous LHRH from the hypothalamus. In previous reports, a change in plasma LHRH was not correlated with spontaneous or $E_2\beta$-induced LH surges in sheep (17, 20). Differences in methodology may partially explain these dissimilar results. The procedure employed in the present experiments to extract LHRH from blood plasma prior to assay differed from that used by Jonas *et al.* (17), whereas Nett *et al.* (20) did not use any extraction procedure. Moreover, the endogenous immunoreactivity measured by Jonas *et al.* (17) was shown to be different from exogenous LHRH. In this regard, Jeffcoate and Holland (21) reported chromatographic evidence that LHRH immunoreactivity in the peripheral blood of the sheep could be separated into multiple peaks. Interestingly, none of the LHRH peaks resolved chromatographically by these authors corresponded to exogenous LHRH. This suggests that the interaction of LHRH with the pituitary may modify the conformation or composition of LHRH. Therefore, the significance of plasma LHRH measurements made by using antisera against synthetic LHRH is unclear.

Summary. Cerebrospinal fluid (CSF) was collected repetitively from the third ventricle of three ovariectomized sheep. Immunoreactive LHRH could not be detected (<100 pg/ml) in any of 70 samples of CSF collected from untreated, estradiol-treated, and LHRH-treated ewes. The LHRH immunoassay accurately measured the hormone in extracts of ovine stalk-median eminence tissue. Furthermore, no immunoreactive LHRH was lost when synthetic LHRH was added to freshly collected CSF and stored in the manner used for the attempted measurement of endogenous LHRH. Intravenous injection of LHRH increased plasma concentrations of the hormone to 420 pg/ml but LHRH could not be detected (<100 pg/ml) in CSF collected concurrently.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 224-227 (1977)

Comparison of Leukocytic Pyrogen and Leukocytic Endogenous Mediator (39642)

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Recent studies (1-3) have demonstrated a variety of biological effects after injecting a crude leukocyte supernatant. Among these effects are lowered plasma iron and plasma zinc, elevated fibrinogen, fever, and an increased number of peripheral blood neutrophils. The causative agent for these effects was designated leukocytic endogenous mediator, LEM (4). Further characterization of LEM revealed many similarities to the leukocytic pyrogen molecule (1, 5) which was known to be present in the crude material. In addition to causing fever, both molecules are proteins, have similar molecular weights (6), and require a free sulfhydryl group for activity. These similarities led us to suspect that LEM and leukocytic pyrogen were the same molecule.

Murphy *et al.* (7, 8) have reported a purification scheme for rabbit leukocytic pyrogen which yields a homogenous product of very high specific activity. We have utilized Murphy's methods with slight modification for preparing and purifying leukocytic pyrogen. Throughout the procedure we tested to see if the LEM activity would be lost as the leukocytic pyrogen became more pure. Only a small amount of highly active protein can be recovered from a large volume of crude material (8); our efforts were, therefore, concentrated on comparisons of biological activities rather than on proving homogenity of the final product.

Materials and methods. Animals. Holtzman-derived rats weighing 180-300 g were maintained at 23° with 12 hr of light and 12 hr of darkness. They were fed Rockland mouse and rat diet and water *ad libitum*. New Zealand white rabbits weighing 2-4 kg were used for temperature assay.

Biological activity. Heparinized plasma was obtained via cardiac puncture 8 hr after ip injection of the test substance. Plasma iron and zinc were determined in deproteinized samples by atomic absorption spectroscopy on a Model 403 Perkin-Elmer spectrophotometer. The total blood neutrophils were determined by diluting blood with Turk's diluting fluid and counting total leukocytes in a hemocytometer, followed by a 100-cell differential count of a smear stained with Wright's stain. Fibrinogen determinations were made 24 hr after injection of the test material using a heat turbidity method (9). Protein determinations were made by the method of Lowry *et al.* (10).

Preparation and purification of leukocytic pyrogen. Each batch of leukocytic pyrogen was prepared from the peritoneal granulocytes of 20 rabbits using the methods and special precautions of Kaiser and Wood (11). This material was then purified by the methods of Murphy *et al.* (7, 8) with two modifications. The first modification was that the crude material was extracted with butanol and methanol (12) and stored as a lyophilized powder instead of in a liquid state. The second modification was the use of polyacrylamide isoelectric focusing instead of sucrose gradient isoelectric focusing. The isoelectric focusing was performed at 4° in a water-cooled electrophoresis apparatus (Hoefer Scientific Instruments, Model EF301). The gels were prepared according to the method of Wrigley as described by Haglund (13). Carrier ampholines, pH range of 3 to 10, were obtained from LKB Produkter AB, Bromma, Sweden. The leukocytic pyrogen was incorporated into the gel and focused for a minimum of 10 hr. The gels were then sliced into 2-mm sections, and each section was eluted overnight in acetate buffer containing 0.1% mercaptoethanol. The eluate was dialyzed against Tris-buffered saline for 2 hr before injection.

After each purification step the leukocytic pyrogen was tested for pyrogenic activity in rabbits and for plasma iron, plasma zinc, neutrophil, and fibrinogen activity (LEM

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ISSN 0037-9727

activity) in rats. In addition, each fraction from the various columns was tested for all activities before pooling.

Results. The yields and specific activities of a typical purification of leukocytic pyrogen at the various stages are presented in Table I. Yields, after the Sephadex CM step for five separate preparations, ranged from 4 to 31%. The percentage of yield was based on the total units of fever-producing activity recovered. After each step of the purification, an aliquot of the pooled material was assayed at three different doses, and a log dose-response curve was fitted. The specific activities increased dramatically through the first three steps but, after the fourth step, the protein concentration was too low to measure.

Throughout the purification, each fraction from the respective columns was assayed separately to avoid possible loss of resolution due to pooling of samples. In each case, the fractions containing leukocytic pyrogen activity also contained LEM activity. Conversely, the fractions that did not show leukocytic pyrogen activity did not contain LEM activity. It was also noted that the maximum response for each activity was found in the same fraction. As an example, Fig. 1 shows the responses to the different slices of a polyacrylamide isoelectric focusing gel. All five activities were found in the same area of the gel, with over 70% of the activity in two adjacent 2-mm slices. The pH in this area of the gel ranged from 7.7 to 7.9.

Table II compares the changes produced by the amount of leukocytic pyrogen necessary to cause 1° fever after various stages of purification. To make these comparisons, we determined from a three-point log dose-response curve the dose necessary to give 1° fever and, then, determined the change in the other activities at this dose. No significant changes were observed in the ratios of the various activities during purification. Most of the variation was less than that expected from a doubling of the dose or the

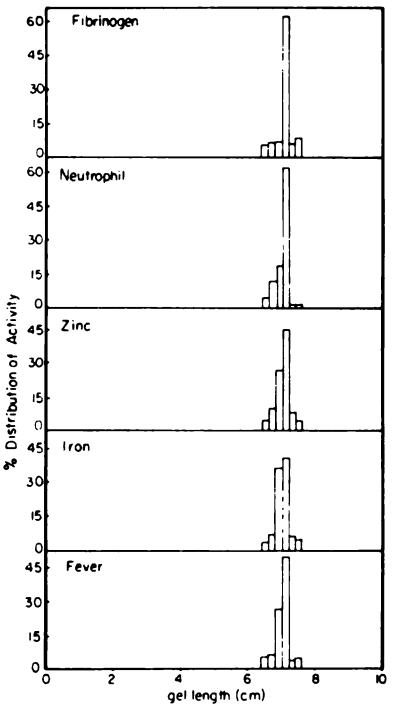

FIG. 1. Effects of polyacrylamide gel isoelectric focusing on the relative distribution of the various biological activities of leukocytic endogenous mediator. Each bar represents the mean response of six animals and is the average from two isoelectric focusing runs. The following changes are equal to 100% activity: fever, 1.56°; plasma iron lowering, 130 μg%; plasma zinc lowering, 89 μg%; increased neutrophil count, 5700/mm³; increased fibrinogen concentration, 158 mg%.

TABLE I. YIELD OF LEM AT VARIOUS STAGES OF PURIFICATION.

Purification step	Protein concentration (mg/ml)	Fever (total units)	Specific activity (units of fever/mg of protein)	Yield (%)
Crude	2.58	2350	2.3	100
Solvent extracted and filtered	0.18	1818	25.2	77
Sephadex G-50	0.01	600	227	25
Sephadex QAE	<0.01[a]	359	–	15
Sephadex CM	<0.01[a]	273	–	12
Isoelectric focusing gel	<0.01[a]	182	–	8

[a] Less than 10 μg/ml as measured by the method of Lowry *et al.* (10).

TABLE II. CHANGES PRODUCED BY THE AMOUNT OF LEM NECESSARY TO CAUSE 1° FEVER.

Purification step	n[a]	Δ Plasma iron (μ/100 ml)	Δ Plasma zinc (μg/100 ml)	Δ Neutrophils (per mm³)	Δ Fibrinogen (mg/100 ml)
Crude	29	96 ± 6[b]	55 ± 4	3753 ± 388	96 ± 7
Solvent extracted and filtered	18	90 ± 5	49 ± 5	4654 ± 671	ND[c]
Sephadex G-50 purified	32	94 ± 6	64 ± 6	4225 ± 534	ND
Sephadex QAE purified	18	95 ± 9	48 ± 6	4580 ± 688	ND
Sephadex CM purified	24	98 ± 7	48 ± 4	4450 ± 600	ND
Isoelectric focusing gel	12	103 ± 12	67 ± 6	3875 ± 490	108 ± 9

[a] Number of animals assayed.
[b] Mean ± SE.
[c] Not determined, ND.

normal biological variation. In the dose-sensitive region of the log dose-response curve, a doubling of the dose caused the following changes: fever, 0.27°; plasma iron, 25 μg%; plasma zinc, 16 μg%; neutrophils, 750/mm³; and fibrinogen, 31 mg%.

Discussion. This purification of leukocytic pyrogen compares favorably with that of Murphy *et al.* (8) through the first three steps where specific activities can be determined. Minor modifications were made in the procedure which should lead to a more homogenous product. The extraction with butanol and methanol was added to remove lipids and other unidentified materials that would interfere with filtration and concentration. This step also stabilizes the crude material; hence, it can be stored without loss of activity. The only other alteration of the procedure was to use polyacrylamide-stabilized gels for isoelectric focusing. No difficulty was experienced with precipitation of proteins during the isoelectric focusing, in contrast to the work of Murphy *et al.* (7) in which urea and dimethylformamide were necessary to prevent precipitation in the sucrose-stabilized system. The isoelectric point of the active material appeared to be in the pH range of 7.7 to 7.9, in agreement with the published value of 7.78 (7). This was not a preparative scale system but it served our purposes adequately, since we began with approximately one-twentieth the amount used by Murphy *et al.* (7) and a larger percentage of the material was consumed by the additional assays before the isoelectric focusing step.

A major concern when purifying a leukocytic pyrogen is to prevent contamination by bacterial endotoxin. In addition to following procedures that minimize this possibility (8, 11), several observations were made indicating that the material purified was not an endotoxin. The active material eluted from the gel filtration columns had a much lower molecular weight than endotoxin and unlike endotoxin passed through an Amicon XM-50 filter. LEM was very labile and lost activity under the relatively mild conditions of isoelectric focusing unless a reducing agent was present. In addition, previous studies in this laboratory have shown that these effects were not due to endotoxin contamination (14–16). Other investigators have also published evidence that LEM is not endotoxin (17).

Five separate batches of leukocytic pyrogen have been purified and tested via this same procedure. In each case, the leukocytic pyrogen and LEM activities migrated as a single unit at every stage of the purification procedure. It appears unlikely that the two proteins would be so closely related that they would have the same molecular weight (6), the same affinities for positively and negatively charged ion-exchange columns, the same migration on disc gel electrophoresis (1), the same isoelectric point, and the same requirement of a free sulfhydryl group for activity (1). It appears that all the effects studied, the changes in body temperature, plasma iron and zinc levels, the number of peripheral blood neutrophils, and the fibrinogen levels are all triggered by a single protein. It would, perhaps, be prudent to withhold final judgment on the possibility of a single molecule being responsible for all of these biological activities until a check can be made with a protein of proven homogenity (8). These results, however, do suggest

that leukocytic pyrogen has many
ıl activities in addition to inducing
Ve prefer the name LEM for this
ng protein, since it indicates the
activities of this mediator.
seems to be a common mediator for
ıs acute phase changes which nor-
company infection or inflammation.
ormation should aid in unraveling
ortant sequence of events and assist
nining their roles in host protection

ary. Leukocytic pyrogen was sub-
o a five-step purification scheme,
ntitative assays for both leukocytic
and leukocytic endogenous media-
M) were made. At each step of the
ion scheme, the active fractions, in
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l zinc concentrations, elevated fi-
ı concentrations, and caused a
of neutrophils from bone marrow. It
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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 228-231 (1977)

Acute Dose Response of Intraocular Pressure to Topical and Oral Cannabinoids (39643)

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The primary active ingredient of marijuana, tetrahydrocannabinol (Δ^9-THC), causes a dose-dependent fall in intraocular pressure (IOP) when given intravenously to man (1) or rabbit (2). In addition, a dose-related fall in IOP is found after THC in sesame oil is applied topically to rabbit eyes (2). However, Δ^9-THC has psychoactive properties which render it unacceptable for widespread clinical use. Recently, newer nitrogen-containing analogs, *N*-substituted benzopyranopyridines, which are structurally related to the cannabinoids, have been synthesized, and have activity in many pharmacological tests similar to the parent compounds (3–5). A spectrum of new compounds has undergone pharmacological and toxicological screening in which some compounds show excellent clinical potential. We have measured the IOP of rabbits and monkeys in response to either a single topical drop or to a single oral dose of each of three of these new drugs to determine their IOP-lowering efficacy. Similar studies with two of these compounds (SP-1 and SP-106), applied topically to rabbit eyes in other vehicles, have been reported previously (6).

Materials and methods. Our studies were made on conscious adult albino rabbits (2 to 4 kg) of either sex. IOP was measured hourly with an Alcon Pneumotonograph, which had been calibrated for the rabbit eye: Determinations were made after application of a drop of 0.5% tetracaine hydrochloride which was washed off after 5 to 10 sec with at least 1 ml of saline solution. One drug, with an *N*-propargyl substitution on the C-ring and a dimethylheptyl branched alkyl side chain, is designated SP-1, is an oil-soluble nitrogen-containing compound structurally related to the cannabinoids (3–6), and was dissolved in a white, light paraffin oil (Fisher Scientific Company). Other benzopyranopyridines, designated SP-106 and SP-204, which are both water-soluble ester derivatives of SP-1 with differing side chains, were dissolved in a commercial artificial tear solution (Ultratears, Alcon Laboratories, Fort Worth, Texas). A baseline IOP was established in each experiment prior to use of the drugs in either rabbits or monkeys by two IOP readings at a 30-min interval: The two values were averaged as the baseline since they differed by less than 1 mm Hg. For topical administration one 50-μl drop of solution was applied unilaterally to one corneal surface, and for oral administration the drug was given in 10 ml of the appropriate vehicle through a stomach tube.

Results. The dose-response curves for the three compounds applied topically in the rabbit are shown in Fig 1. All drugs caused a fall in IOP compared to vehicle alone. The maximum fall in IOP occurred, almost without exception, at 120 min and returned to original baseline IOP at 6 hr with low drug concentrations (0.01%) but with 1% an IOP fall of between 5 to 10% was still present at 6 hr. The contralateral eye also showed a fall in IOP between 1 and 3% less than the treated eye although the time course of action was identical. Vehicle alone caused only a 1 to 3% reduction in IOP.

After oral administration to the rabbit the fall in IOP was not as marked as after topical administration at equivalent dose levels (mg/kg), which difference may represent less complete absorption from the intestine compared to that from orbital tissues. The dose-response curve (Table I) is much less steep than that seen after topical doses (Fig 1). SP-106 caused a more rapid fall in IOP than SP-1 (SP-204 was not tested in these experiments due to a shortage in supply). With SP-106 the effect was complete and baseline IOP regained at 7 hr, whereas SP-1 appeared to have a longer lasting effect which was of slower onset.

Studies in ketamine-immobilized juvenile (3 kg) rhesus monkeys (*M. mulatta*) indi-

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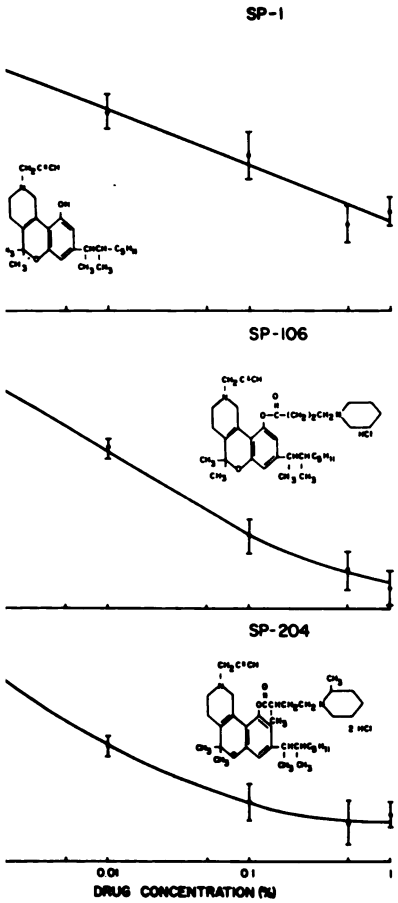
ISSN 0037-9727



Dose response of rabbit intraocular pres-
ree topically applied nitrogen-containing
related to the cannabinoids. Each value is
± the standard error of the mean of the
ntraocular pressure fall for 10 treated eyes
ils.

at 0.1% concentrations of these
ugs caused little change in IOP
olied topically (maximum 7% at 1
of 5 animals, 10 eyes), but 0.5%
fall in IOP (maximum of 20% at 2
out 4 to 5 hr duration (Table II).
lone given to the eye caused only a
1 1% fall in IOP. The regimen used
ame as that in the rabbit studies,
monkeys used as treated and one
rol per test drug. The control ani-
hanged between experimental ses-
nich were a minimum of 3 days
ice the IOP fell in both eyes, the
1 both treated and untreated eyes
oled. The need for a higher dose level in the monkey may be related to a higher metabolism of these compounds compared to the rabbit.

Discussion. The present data indicate that, when given acutely, the newer benzopyranopyridines tested all cause a fall in IOP of both the rabbit and monkey eye. There is a quantitative difference, however, in their effects and this is presumably another reflection of the differences they exert in a variety of tests. Some drugs appear, if side chains are modified, to elicit quantitatively different responses compared to the parent compound. The new compounds seem to demonstrate somewhat selective effects on different parameters (3–6), revealing different pharmacological spectra.

That the IOP falls in both eyes when administered only to one eye establishes the systemic absorption of these drugs, which has been noted previously for Δ^9-THC (7, 8). The difference in response of the contralateral eye is usually small, being between 1 and 3%.

Following oral drug administration more marked differences between the biological responses to the three drugs are seen. The IOP fall after SP-106 is more rapid in onset than that of SP-1 and this may be a reflection of either more rapid absorption of the water-soluble compound or better release of the compound from a water-based rather than a mineral-oil-based vehicle. Previous work on intraocular penetration of Δ^9-THC from different vehicles demonstrated that the release of the drug varied from vehicle to vehicle (9).

The higher concentration required in monkeys may be related to a more rapid metabolism of these compounds, thus higher dose levels of the cannabinoids would be required to produce a biological effect.

All three compounds reduce IOP by acting both locally in the eye (8, 9) and via a systemic route, since an untreated contralateral eye shows a fall in IOP. A locus of action is presumably some central site in the adrenergic nervous pathways (9), since both superior cervical ganglionectomy and preganglionectomy section of the cervical nerve reduce the IOP lowering effects of Δ^9-THC (7–9).

In an experimental animal there is no way

TABLE I. PERCENTAGE REDUCTION IN INTRAOCULAR PRESSURE IN THE RABBIT IN RESPONSE TO ORAL BENZOPYRANOPYRIDINES[a]

	SP-106				SP-1			
Time (min)	0.003 mg/kg	0.03 mg/kg	0.15 mg/kg	0.3 mg/kg	0.003 mg/kg	0.03 mg/kg	0.3 mg/kg	1.5 mg/kg
0[b]	21.9	21.0	21.1	20.6	21.0	21.1	21.0	20.0
60	3	11	12	11	9	10	7	4
120	3	14	12	17	6	15	13	3
180	8	11	12	12	4	10	16	17
240	4	13	12	5	5	14	17	19
300	3	6	9	1	2	10	11	18
360	2	2	3	1	1	5	8	16
420	1	+2	2	0	1	4	2	13

[a] The values represent the IOP fall in 10 normal eyes of 10 rabbits.

[b] The original baseline IOP in mm Hg (−30 min and 0 time prior to drug administration) is given in the first horizontal line. The largest SEM is ±2.3% (i.e., about 20% of the IOP fall, not shown for clarity). Data for the contralateral eye are not given as these eyes had been subjected to superior cervical ganglionectomy and, therefore, the IOP response was much reduced (7–8).

TABLE II. FALL IN INTRAOCULAR PRESSURE OF JUVENILE RHESUS MONKEY AFTER TOPICAL BENZOPYRANOPYRIDINES.[a]

	Percentage reduction in IOP		
Time (min)	SP-1 0.5%	SP-106 0.5%	SP-204 0.5%
0[b]	19.20	19.75	19.75
60	13	12	12
120	17	16	13
180	15	16	11
240	13	11	19
300	5	9	13
360	1	3	11

[a] The values represent the IOP fall in at least eight eyes, the data from both eyes is used since the IOP fall in the untreated eye differed little from that in the treated eye.

[b] The original baseline IOP in mm Hg (−30 min and 0 time, prior to drug administration) is given in the first horizontal line. The largest SEM is ±2.5%.

to discern whether or not the euphoric/dysphoric effects of these drugs are revealed and this will only be determined by testing in man. Preliminary data (10) indicate that dose levels greater than 0.06 to 0.1 mg/kg of SP-106 when given orally were required to elicit any noticeable subjective effects in human volunteers. This compares to the present studies where dose levels of 0.01 mg/kg (topical in rabbit), 0.08 mg/kg (topical in monkey), or 0.02 mg/kg (oral in rabbit) were found to produce pronounced IOP reductions. If further testing shows that the psychoactive properties of these compounds are found to be minimal or absent at dose levels required to reduce IOP then these compounds may be of some value clinically to reduce elevated IOP. The IOP fall is as good or better than presently available drugs (11) and the benzopyranopyridines appear to offer a favorable alternative to current topical therapy.

Summary. Three nitrogen-containing cannabinoids caused a dose-dependent fall of intraocular pressure when administered either topically or orally to rabbits. Concentrations of 0.001 to 1% applied topically and 0.3 mg/kg orally were effective. Topical concentrations of 0.5% caused a substantial decrease of intraocular pressure in rhesus monkeys. These compounds may prove therapeutically useful in the treatment of elevated intraocular pressure.

This work was supported in part by PHS Research Grants EY 01413, EY 01590 and DA 01213. We thank Dr. Harry Pars of SISA, Inc., Cambridge, Mass., for the generous supplies of the benzopyranopyridines, Ms. D. Hancock and Mrs. I. Prior for secretarial assistance, Ms. K. Bowman and Ms. D. Johnson for technical assistance, and Drs. M. Kling, R. Shimp and Mr. R. Morrison for their help with the monkey experiments.

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Received September 8, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 232-237 (1977)

Renin Secretion after Papaverine and Furosemide in Conscious Sheep[1] (39644)

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Papaverine has proven to be a useful pharmacological tool to study renin secretion because it blocks the intrarenal vascular receptor that regulates renin release (1). Results of studies in which papaverine has been used in conjunction with the nonfiltering kidney model (2) have favored a predominant role for the vascular receptor in regulating renin secretion (3). These studies have been performed exclusively in anesthetized animals, usually dogs, and data relating to the effects of papaverine on renin secretion and renal function in conscious animals are lacking.

Papaverine induced blockade of the renal vasculature should also provide a useful means for examining the function of the macula densa in regulating renin release. Furosemide-stimulated renin release during isovolemia has been taken as evidence for a functional role of the macula densa (4), but, furosemide is well known to produce hemodynamic changes within the kidney (5). These changes in vascular tone might increase renin secretion by a direct effect on the intrarenal baroreceptor (6). If renin secretion could be stimulated by furosemide in the presence of vascular blockade with papaverine, it would support the concept of a role for the macula densa in regulating renin release.

The present studies were undertaken to define the effects of papaverine in conscious animals and to determine if furosemide would stimulate renin secretion during renal arterial infusion of papaverine and maintenance of constant extracellular volume.

Methods. Six mixed breed sheep (25-40 kg body wt) underwent a right nephrectomy 3-6 weeks before experimentation. One to three days before the animals were to be studied, anesthesia was induced with intravenous pentothal and maintained with halothane-O_2 for implantation of chronically indwelling catheters. The left renal artery was catheterized by the Herd-Barger technique (7) using Silastic tubing (Dow-Corning Co.), and a noncannulating electromagnetic flow probe (Carolina Medical Electronics) was placed around the renal artery at its origin. The flow probe had been calibrated previously *in vitro* using whole blood perfused through segments of sheep carotid arteries. Another Silastic catheter was introduced via a small tributary into the renal vein. The ureter was catheterized with polyvinyl chloride (PVC) tubing close to the renal pelvis. Additional PVC catheters were placed in a carotid artery via a transverse facial artery and into a jugular or saphenous vein. The vascular catheters were maintained patent by daily flushing with 100 U/ml heparin-saline solution.

The experiments were conducted with the animals standing quietly in their cages. Intravenous inulin was continuously administered during the experimental procedure to determine glomerular filtration rate. Zero renal blood flow was determined electronically and both mean flow and arterial pressure were recorded on a Gilson recorder. During the experiment, all urine volumes above control level were replaced continuously with sterile normal saline delivered by a roller pump (Gilson, Minipuls) into a jugular or saphenous vein catheter. All blood samples were immediately replaced with fresh whole blood from another sheep.

Two experimental protocols were followed: To determine the effects of papaverine alone on renin secretion in conscious sheep, saline was first administered into the renal artery at 0.6 ml/min and two 10-min clearance periods were performed. Simultaneous systemic arterial and renal venous blood samples were obtained at the midpoint of each clearance period. Subsequently, the renal arterial infusion was

[1] This work was supported by Grant HL 14683 from the National Institutes of Health.

[2] Recipient of USPHS Research Career Development Award HL 00003.

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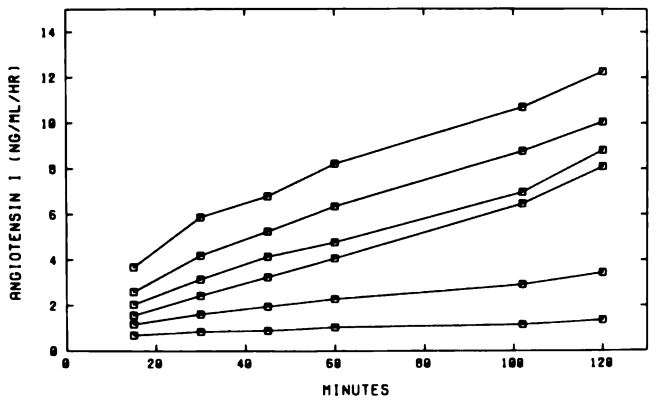
ISSN 0037-9727

d to papaverine in isotonic saline and ed at 7 mg/min and two further clear- ›eriods were performed. In some the papaverine infusion was in- l to 10 mg/min for one or two addi- :learance periods. The second proto- ; similar to that just described except 'ter two or three clearance periods nal intra-arterial infusion of papaver- mg/min), 35 mg of furosemide (La- s administered intravenously over 10 Three further clearance determina- /ere made at 20-min intervals with rine continuously infused into the rtery.

na renin activity was determined by ımunoassay of *in vitro* generated an- in I based on the technique of Haber :agents were supplied in kit form by ngland Nuclear Co. The incubation rried out for 1 hr at pH 6.0 without n of substrate. Aliquots of all plasma s were maintained at 4°C and assayed neously with the incubated samples. munoreactive material assayed in the bated sample was substracted from asured angiotensin I in the incubated to yield the value for arterial and enous plasma renin activities. Figure rates that the angiotensin I produc- ıs linear for up to 2 hr of incubation t added substrate for a wide range of renin activities. Renin secretion was ted by multiplying the renal venous arterial plasma renin activity by the renal plasma flow.

Plasma and urinary electrolytes were determined by flame photometry. Mean arterial blood pressure and mean renal blood flow were determined by sampling these variables once each minute and averaging over the 10- or 20-min clearance period. Inulin in plasma and urine was determined by the method of Heyrovsky (9). Statistical analysis was carried out on a pair design basis utilizing Student's *t* test.

Results. Effect of papaverine alone on renin secretion. Twelve experiments were conducted on different days on six conscious sheep. The animals stood or laid quietly in their cages during the entire experiment. The only behavioral effect discernible during papaverine administration was licking of the cage, which generally occurred toward the end of the experimental period. Because urine flow never exceeded control levels during the administration of papaverine only, replacement of urine volume was not undertaken in these experiments. Table I presents data obtained with normal saline or 7 or 10 mg/min of papaverine in a 0.9% NaCl solution infused into the renal artery at 0.6 ml/min. Papaverine at 7 mg/min produced a slight but statistically significant fall ($P < 0.01$) in mean arterial blood pressure in the conscious sheep. Likewise, there was a significant increase ($P < 0.01$) in mean renal plasma flow (RPF) reflecting the decrease in renal resistance secondary to papaverine infusion. Glomerular filtration rate



1. *In vitro* generation of angiotensin I from sheep plasma for several levels of renin activity. The ›n was conducted at 37° for 2 hr to illustrate the linearity of the response without addition of exogenous :. The experimental samples for this study were incubated for 60 min and are within the range of renin illustrated.

(GFR) fell significantly ($P < 0.01$) resulting in a decrease in the filtered load of Na ($P < 0.01$). Urinary Na excretion and the percentage of filtered Na excreted did not change. Increasing the renal arterial infusion rate of papaverine to 10 mg/min produced no further increase in RPF. In fact, at this level of papaverine administration, there was a slight but significant ($P < 0.05$) decrease in RPF in each animal studied. A further decrease in GFR was observed ($P < 0.05$) but no changes in other measured variables when compared to the 7-mg/min papaverine infusion.

Figure 2 illustrates the changes in renin secretion for each animal in this experiment. No statistically significant changes in renin secretion occurred with either 7 or 10 mg/min of papaverine when compared to the saline control values. In three instances during renal arterial infusion of saline or papaverine, renin was being taken up by the kidney rather than being secreted. These data suggest intermittent secretion of renin in conscious sheep but the observations are too limited to suggest possible significance or controlling factors.

Effects of papaverine and furosemide on renin secretion. The results obtained after intravenous injection of 35 mg of furosemide in conscious sheep which were undergoing a renal arterial infusion of papaverine are illustrated in Fig. 3 and Table II. In these animals urine volume above control levels was continuously replaced by an intravenous saline infusion. Arterial blood pressure and RPF were not significantly different from the papaverine only control period after furosemide administration. GFR was significantly different only during the final 20-min clearance period. Renal sodium excretion increased rapidly and was elevated significantly at each clearance period after furosemide administration. Likewise, large statistically significant increases in renin secretion paralleled the increase in Na excretion.

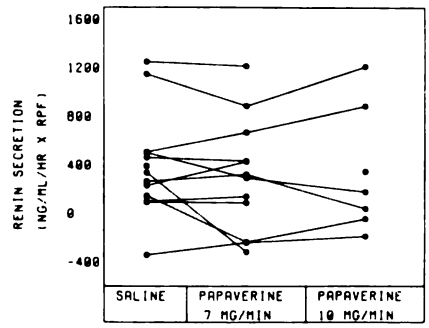


FIG. 2. Individual animal renin secretion rates during renal arterial infusion of normal saline or 7 or 10 mg/min of papaverine. All infusion were at 0.6 ml/min. ●, the mean for each treatment group with the negative values included. No statistical differences exist between groups whether the negative values are considered as such or whether they are considered 0 secretion.

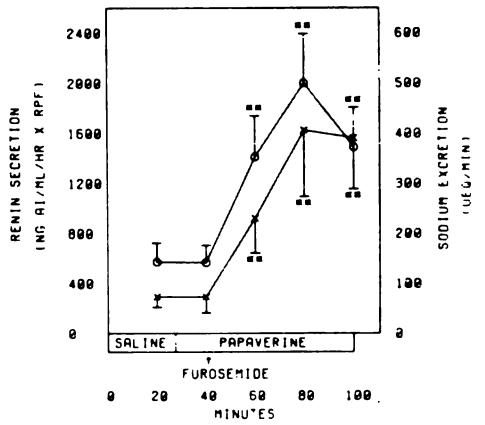


FIG. 3. Renin secretion (O——O) and Na excretion (×——×) during renal arterial infusion of saline or papaverine (7 mg/min) and after an intravenous injection of 35 mg of furosemide with papaverine continuously infused into the renal artery. All renal arterial infusions were at 0.6 ml/min. Statistical analysis is based on the difference between the papaverine alone period and the periods after furosemide injection. Mean ± SEM, ** = $P < 0.01$, $N = 5$.

Discussion. The present experiments were conducted in conscious animals to eliminate the effect of anesthesia on renin secretion (10, 11). Additionally, it was noted that papaverine infused into the renal artery did not increase Na excretion as has been previously reported in experiments on anesthetized dogs (12, 13). The explanation for this discrepancy between the effects of papaverine on renal Na handling in conscious sheep and anesthetized dogs is not clear. We have recently studied renal arterial infusion of papaverine in anesthetized sheep and observed increased Na excretion similar to that reported for anesthetized dogs (unpublished). It is assumed that the decreased filtered load of Na which results from the decrease in GFR would result in a

TABLE I. RENAL FUNCTION AND ARTERIAL BLOOD PRESSURE DURING INFUSION OF NORMAL SALINE OR PAPAVERINE INTO THE RENAL ARTERY.[a]

	Saline	Papaverine	
		7 mg/min	10 mg/min
BP (mmHg)	93 ± 5[b]	90 ± 5	93 ± 6
		(−3 ± 0.7*)[c]	(0.3 ± 1.0)
RPF (ml/min)	438 ± 30	462 ± 22	442 ± 24
		(40 ± 10**)	(−32 ± 15*)
GFR (ml/min)	34 ± 3	28 ± 3	21 ± 4
		(−7 ± 1**)	(−4 ± 2*)
Filtered Na (μEq/min)	5072 ± 407	4098 ± 405	3227 ± 349
		(−975 ± 254**)	(−482 ± 312)
$U_{Na}V$ (μEq/min)	80 ± 24	75 ± 21	79 ± 26
		(−4 ± 15)	(−32 ± 25)
Filtered Na excreted (%)	1.6 ± 0.4	1.8 ± 0.5	2.4 ± 0.6
		(0.4 ± 0.3)	(0.4 ± 0.5)
N	12	12	6

[a] All renal arterial infusions at 0.6 ml/min. Statistical analysis compares the difference between papaverine (7 ng/min) and saline alone and the difference between 10- and 7-mg/min papaverine infusions.
[b] Mean ± SEM.
[c] Mean difference ± SEM difference.

TABLE II. RENAL FUNCTION AND ARTERIAL BLOOD PRESSURE DURING RENAL ARTERIAL INFUSION OF SALINE OR PAPAVERINE AND AFTER INTRAVENOUS FUROSEMIDE.[a]

	Saline 20 min	Papaverine 40 min	Papaverine and furosemide		
			60 min	80 min	100 min
BP (min Hg)	85 ± 8[b]	82 ± 7	92 ± 12	90 ± 10	92 ± 11
			(10 ± 5)[c]	(4 ± 2)	(6 ± 3)
RPF (ml/min)	393 ± 52	433 ± 377	395 ± 48	407 ± 35	411 ± 44
			(−38 ± 28)	(−26 ± 33)	(−22 ± 31)
GFR (ml/min)	31 ± 4	23 ± 3	24 ± 2	19 ± 3	18 ± 3
			(1 ± 4)	(−4 ± 4)	(−5 ± 2*)
iltered Na (μEq/min)	4603 ± 514	3421 ± 391	3654 ± 4077	2920 ± 508	2742 ± 423
			(233 ± 622)	(−493 ± 681)	(−679 ± 270*)
$_{Na}V$ (μEq/min)	73 ± 21	74 ± 33	230 ± 69	406 ± 132	390 ± 101
			(157 ± 40**)	(333 ± 103**)	(317 ± 71**)
iltered Na excreted (%)	1.9 ± 0.7	2.3 ± 1.0	7.8 ± 3.5	14.3 ± 3.7	14.9 ± 3.1
			(5.4 ± 1.8**)	(12.0 ± 3.0**)	12.5 ± 2.3**

[a] Saline was administered into the renal artery at 0.6 ml/min for the first 20 min; then the infusion was changed to papaverine (7 mg/min) in normal saline for the duration of the epxeriment. Furosemide (35 mg) was injected slowly as a single bolus at 60 min. Statistical analysis compares each of the three periods after furosemide injection to the period during which only papaverine was infused. * = $P < 0.05$, ** = $P < 0.01$, $N = 5$.
[b] Mean ± SEM.
[c] Mean difference ± SEM difference.

decrease in Na excretion if fractional reabsorption remained constant or nearly so. In fact, in 8 of the 12 experiments conducted with papaverine alone infused into the renal artery, Na excretion did decrease. It appears that any inhibitory effect papaverine might have on Na reabsorption by the nephron is augmented considerably by anesthesia.

As postulated by Vander and Carlson (4), decrease in the filtered load of Na should be reflected in a decreased load of Na at the macula densa and result in an increased renin secretion. In the present experiments papaverine alone resulted in a 19% decrease in filtered Na but no increase in renin secretion was observed. This observation could be interpreted to suggest that small changes in filtered load of Na do not affect the macula densa receptor. However, lacking specific information on proximal tubular reabsorption of Na under the influence of

papaverine (*vide supra*), it is difficult to draw a firm conclusion regarding the Na load presented to the macula densa. It is possible that proximal reabsorption decreased sufficiently to allow no change in macula densa Na load despite the decreased filtered load of Na.

The reasoning behind combining papaverine and furosemide to study the role of the macula densa is that by blocking vasomotion of the renal vasculature and maintaining renal perfusion pressure constant, any influence on renin secretion by the vascular receptor mechanism would be minimized. If alterations in NaCl transport by the macula densa segment can be effected under these conditions, it would be reasonable to assume that any renin secretion which occurred would be due to alterations in ion transport and not secondary to pressure changes.

The elevated renin secretion observed after furosemide administration was not associated with changes in arterial blood pressure or renal blood flow. Urinary Na excretion rose markedly and closely paralled the increase in renin secretion. These data suggest that the stimulated renin secretion under these conditions results from alterations in tubular ion transport and not from changes in vascular tone or distending pressure at the baroreceptor.

Corsini *et al.* (14) recently reported that furosemide stimulated renin secretion in filtering and nonfiltering kidneys of anesthetized dogs. Papaverine blocked this response in the nonfiltering kidney but renal vasodilation by acetylcholine did not block the response in filtering kidneys. Papaverine was not studied in this context in dogs with filtering kidneys. Although the data of Corsini *et al.* would suggest that part of the furosemide-stimulated renin release was secondary to intrarenal baroreceptor activation, their data using acetylcholine induced renal vasodilation and the results from this study would support the concept of a role for the macula densa in regulating renin secretion.

Because these animals did not undergo renal denervation or adrenalectomy, it is possible that the renal nerves or circulating catecholamines could have influenced renin secretion during furosemide stimulation. This possibility would appear unlikely, however, because volume depletion was prevented by replacing urinary and blood losses. Also, Johnson *et al.* (15) have demonstrated that papaverine blocks the stimulatory effect of epinephrine on renin secretion.

These studies do not indicate the nature of the signal perceived by the renal tubular mechanism after furosemide administration; however, since furosemide inhibits transcellular NaCl transport it is likely that this is the variable being sensed. The recent observation by Burg and co-workers (16) and Kokko and co-workers (17) that Cl is the actively transported ion in the ascending limb of the loop of Henle and that furosemide inhibits active Cl transport by this segment, suggest that the macula densa mechanism might be Cl-sensitive rather than Na-sensitive. This is supported by our earlier suggestion (6) that the macula densa probably transports NaCl in a manner similar to the ascending limb of the loop of Henle, since renin secretion is not inhibited by aldosterone (18).

Summary. Conscious sheep with chronically implanted vascular catheters and a renal artery flow probe were used to study the effects of papaverine alone or papaverine plus furosemide on renin secretion. Papaverine infused into the renal artery at 7 mg/min produced a significant renal vasodilation which was not further enhanced by increasing the papaverine infusion to 10 mg/min. GFR decreased significantly as did the filtered load of Na. The increased Na excretion reported in anesthetized dogs was not observed in conscious sheep. Renin secretion was not altered despite these changes in renal function. When 35 mg of furosemide was administered intravenously in addition to the renal artery papaverine infusion, no further change in RPF or GFR was observed. Na excretion was significantly elevated at each clearance period after furosemide administration and these increases were closely paralleled in time by large increases in renin secretion. The increased renin secretion observed from the papaverine blocked kidney after furosemide administration appears to be mediated by changes

in ion transport by the renal tubules and not by activation of the intrarenal baroreceptor.

The author expresses his gratitude to Hoechst-Roussel Pharmaceutical Corporation for the furosemide used in this study and to Miss Maureen Garver for her expert technical assistance

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Received July 20, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 238-241 (1977)

Dose-Response Relationships between Glucocorticoids and Growth Inhibition in Rat Glioma Monolayer Cultures[1] (39645)

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Cell proliferation is inhibited when log phase rat glioma (strain C6) monolayer cultures are treated with either 3 μM cortisol or 0.01 μM dexamethasone (1, 2). We now report that the suppression of C6 growth is dose-dependent over a wide range of glucocorticoid concentrations.

Methods and Materials.[2] C6 cells (3) were grown at 37° in Kimble plastic tissue culture flasks (25 cm^2) containing 5 ml of medium. The culture medium consisted of Ham's F10 Nutrient Mixture (4) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES). The pH of the medium was adjusted to 7.4 with 1 N NaOH. Cortisol and dexamethasone stock solutions (10 mM) were prepared by dissolving the glucocorticoids in 50% ethanol-50% phosphate-buffered saline (PBS, pH 7.4). After filtration (0.22 nm), these solutions were diluted into fresh medium at 37° to achieve final glucocorticoid concentrations ranging from 0.0001 to 100 μM. Fluorometric analysis (5) of the fetal calf serum revealed that the control culture medium contained the equivalent of 0.003 μM cortisol.

Cell densities were measured with a hemacytometer. To determine the accuracy and precision of this method, a C6 cell suspension was diluted serially and over 300 cells were counted in quadruplicate from each dilution. Mean cell densities/ml were calculated and a least-squares linear regression analysis was employed to fit a curve which spanned ~2 orders of magnitude. The standard errors (SE) of the means were <7% and the correlation coefficient was >0.99.

The experiments were initiated by inoculating culture flasks containing 4.5 ml of medium with 0.5-ml aliquots from a C6 cell suspension to achieve 2-3 × 10^4 cells/cm^2. The cultures were divided into several groups on the following day (Day 0). One group served as the control and received fresh medium. The remaining groups were treated with fresh medium containing either cortisol or dexamethasone. The ethanol concentration was adjusted to 0.05% in control and treated cultures; the log phase growth rate in control cultures was not altered in the presence of 0.05% ethanol. Growth was monitored in control and glucocorticoid-treated cultures on Days 0, 1, 2, 4, and 6 by measuring mean cell densities (mean cell number/cm^2). For each measurement, quadruplicate monolayers from each group were washed 3× with PBS and the cells were dispersed by the action of 0.25% trypsin. The cells were counted with a hemacytometer and the mean cell densities (±SE) were calculated. The Day 0 mean cell denity was subtracted from the mean cell densities measured on Days 1, 2, 4, and 6. These values were used to calculate the percent of control growth which occurred in the glucocorticoid-treated cultures since Day 0.

Results. The growth-inhibitory effects elicited by cortisol and dexamethasone in C6 cultures are expressed in the form of dose-response curves (Fig. 1). These curves were generated from mean cell density data compiled from 15 separate experiments.

[1] This work was supported by grants from Merck Sharp and Dohme Research Laboratories (West Point, Pa.) and the American Cancer Society-Florida Division (No. F-72-SF-1).

[2] The materials used in this study were purchased from the following suppliers: C6 cells (American Type Culture Collection, CCL 107, Rockville, Md.); Ham's F10 Nutrient Mixture (Pacific Biologicals, Berkeley, Calif.); fetal calf serum and trypsin (Grand Island Biological Co., Grand Island, N.Y.); antibiotics (International Scientific Industries, Cary, Ill.); HEPES (Calbiochem, La Jolla, Calif.); cortisol (Sigma Chemical Co., St. Louis, Mo.). Dexamethasone was a generous gift from Merck Sharp and Dohme (West Point, Pa.).

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ISSN 0037-9727

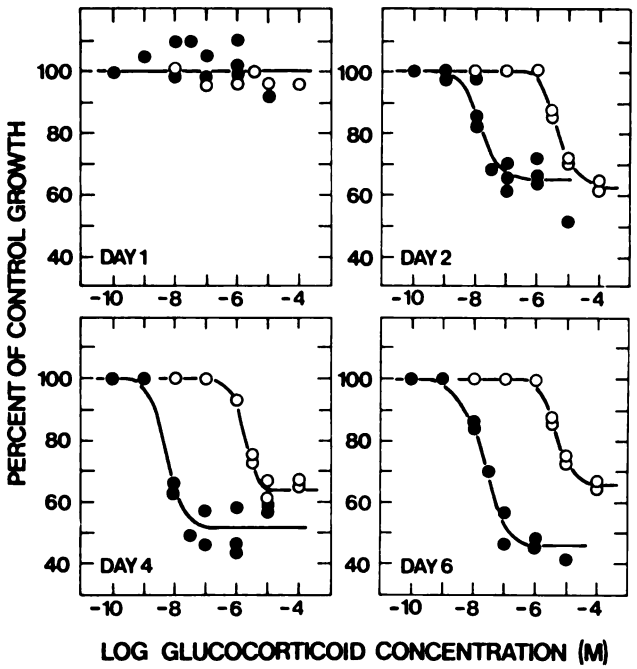

FIG. 1. Dose-response relationships between glucocorticoid concentrations and inhibition of rat glioma (strain C6) cell proliferation. Several concentrations show multiple symbols which represent data from separate experiments. The SE ranges which were <7% were omitted from the figure for clarity. Cortisol, ○; dexamethasone, ●.

Their salient features are as follows:

Day 1: The control population doubled every 20 hr during log phase. Mean cell densities in C6 cultures treated for 1 day with either cortisol (0.01-100 μM) or dexamethasone (0.0001-10 μM) were similar to controls. Cell proliferation continued in these treated cultures at log phase rates. Over a wide concentration range, neither glucocorticoid exerted an immediate growth inhibitory effect on C6 cell proliferation.

Day 2: Control cells continued to divide every 20 hr. Relative to controls on Day 2, mean cell densities were ~35% lower in C6 cultures treated with 100 μM cortisol or >0.1 μM dexamethasone. The dose-response curve for cortisol revealed that the ED_{50} (i.e., the effective concentration that produces 50% of this maximum response) is ~3 μM. In contrast, the ED_{50} for dexamethasone was ~0.01 μM. Thus, dexamethasone is 250 to 300 times more potent than cortisol in suppressing C6 cell proliferation.

Day 4: The rate of growth in control cultures decreased between Days 2 and 4, resulting in stationary phase cell densities of ~1.5 × 10^5 cells/cm^2. Growth decreased at a similar rate in the lower cell density cultures treated with 100 μM cortisol; the maximum inhibitory response at this cortisol concentration on Day 4 was not significantly different from that measured on Day 2. In C6 cultures treated with 3 μM cortisol, the inhibitory response increased from 14% on Day 2 to 25% on Day 4. The maximum inhibitory response was achieved at 100 μM cortisol by Day 2, but a period longer than 2 days was required for 3 μM cortisol to elicit a response which approached maximum inhibition.

In contrast to the effect of 100 μM cortisol, C6 cells treated with >0.1 μM dexamethasone failed to proliferate after Day 2. As a result, the maximum growth-inhibitory response increased from 35% on Day 2 to 50% on Day 4 as control cell populations approached stationary phase. In addition to its greater potency, dexamethasone appears to be ~1.5× more efficaceous than cortisol in suppressing C6 growth. The inhibitory response exerted by 0.01 μM dexa-

methasone increased from 17% on Day 2 to 35% on Day 4. At 0.03 μM dexamethasone, the response increased from 28 to 46% during the same period. Similar to 3 μM cortisol, more than 2 days were required for 0.01–0.03 μM dexamethasone to elicit a response approaching maximum growth inhibition.

Day 6: Between Days 4 and 6, no cell proliferation occurred in the stationary phase control cultures or in the lower cell density cultures treated with either 100 μM cortisol or >0.1 μM dexamethasone. However, mean cell densities increased during this period in C6 cultures exposed to lower concentrations of both glucocorticoids. The growth-inhibitory response decreased from 26 to 13% and from 38 to 25% in cultures treated with 3 μM and 10 μM cortisol, respectively. The response decreased from 35 to 15% and from 46 to 30% in cultures exposed to 0.01 and 0.03 μM dexamethasone, respectively. These observations confirm and extend previous findings (1, 2) that cortisol and dexamethasone at these concentrations elicit a transient growth-inhibitory response in C6 cultures.

Phase contrast microscopy did not reveal morphological differences between control and glucocorticoid-treated C6 cells. No detached cells were observed floating in the medium. Control and glucocorticoid-treated cells excluded erythrosin B dye equally well (>92%). The reduction in mean cell densities in treated cultures relative to controls does not appear to result from killing a fraction of the cell population by steroidal cytotoxicity.

Discussion. The growth-inhibitory response elicited by cortisol and dexamethasone in C6 cultures reflects their relative potencies as anti-inflammatory agents (6). Similar relationships between different glucocorticoids and their ability to suppress log phase growth have also been observed in murine L-929 (7–10), lymphoma ML-388 (11), and lymphoma L-5178 Y (12, 13) cell cultures. However, the transient growth-inhibitory responses that occur in glucocorticoid-treated C6 cultures have not been demonstrated with these murine cell lines.

Glycerol phosphate dehydrogenase activity is induced in C6 cells at glucocorticoid concentrations similar to those used in this study (14, 15). This enzymatic activity also appears to be regulated by glucocorticoids in differentiating neonatal rat brain (16) and begins to accumulate at the same time glia cease to proliferate (17). Thus, the mechanisms which regulate cell proliferation and enzyme induction in C6 cells may share common components.

Growth inhibition occurs in C6 cultures treated with glucocorticoids at concentrations considered chemotherapeutic *in vivo*. Only two *in vivo* studies ascribe prolonged survival times to glucocorticoid action in treated animals bearing glioma cells implanted intracerebrally. Prolonged survival was accompanied by decreases in tumor size after rats harboring implanted rat glioma cells were treated with methylprednisolone (18) and mice bearing murine ependymoblastoma cells were treated with dexamethasone (19). In addition to the anti-edema effects exerted by glucocorticoids (20, 21), the reduction in glioma size may also be related to the inhibition of cell proliferation described in this report.

The C6 cell line retains many morphological and differentiated glial characteristics (14, 15, 22–26) and continues to respond to the growth-inhibitory effects exerted by glucocorticoids after prolonged cultivation (2). Hence, homogeneous C6 cell populations may serve as a suitable model system for investigating pharmacological interactions between anti-tumor agents and glioma physiology both *in vitro* and *in vivo*. Dexamethasone-treated C6 cultures could be employed to test other chemotherapeutic agents that penetrate the blood–brain barrier for their ability to potentiate the growth-inhibitory response elicited by the glucocorticoid. We have recently discovered that combined growth-inhibitory responses occur in C6 cultures treated with 1 μM dexamethasone and 1,3-bis(2-chloroethyl)-1-nitrosourea over a wide concentration range (27). Together with *in vivo* studies utilizing C6 cells implanted into rat brain, this approach may suggest chemotherapeutic regimens to suppress glioma growth at drug concentrations in which host toxicity is minimized or eliminated.

Summary. Cell proliferation is inhibited

ı rat glioma (strain C6) monolayer cultures reated with cortisol (>1 μM) or dexamethsone (>0.001 μM). Transient growth-inibitory responses occur at 3 μM cortisol nd 0.01–0.03 μM dexamethasone. At igher glucocorticoid concentrations, nonransient inhibitory responses persist for 5 ays. Dose–response curves show that dexaıethasone is 250 to 300 times more potent han cortisol in suppressing C6 growth.

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Received July 15, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 242-245 (1977)

Increased Responsiveness to LH-Releasing Hormone (LHRH) in Rats with Median Eminence Lesions (39646)

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Lesions in the median eminence of the tuber cinereum inhibit the release and synthesis of LH as evidenced by declines in plasma and pituitary LH measured by both bio- and radioimmunoassay (1-4). The inhibition of LH release and synthesis is presumably caused by the destruction of the neurosecretory system which discharges LH-releasing hormone (LHRH) into hypophyseal portal vessels to stimulate LH release in the intact animal. Alternatively, damage to hypophyseal portal vessels produced by the lesions could reduce adenohypophyseal blood flow which could interfere with the function of gonadotropes and lead to diminished hormone release. The present studies were carried out to determine the responsiveness of the adenohypophysis to synthetic LHRH at various times after median eminence lesions in orchidectomized male rats. To minimize possible damage to the hypophyseal portal vessels, discrete radiofrequency lesions were placed bilaterally in the anterior median eminence.

Materials and methods. Adult male rats weighing approximately 250 g of the Sprague-Dawley strain (Simonsen Laboratories, Gilroy, California) were used. They were fed Purina laboratory chow and given free access to tap water. Rats were caged in group cages in a room with controlled temperature (22°) and lighting (lights on 500-1900 hr). Castrated animals were utilized 1 month after orchidectomy.

Bilateral radiofrequency lesions were placed in the median eminence using a radiofrequency lesion maker (Grass mono LM-3; intensity, 90; duration, 30 sec) as previously described (3). Stainless steel wires 0.3 mm in diameter and insulated except for the flattened tip were used. In sham-operated rats the electrode was lowered 5 mm below the dura without passage of current. One-milliliter heparinized blood samples were removed from the external jugular vein while the animals were lightly anesthetized with tribromoethanol (5). Blood was taken just prior to placement of lesions and just before and at 10 and 20 min after the injection of synthetic LHRH on the 1st, 7th, and 14th post-operative days. Synthetic LHRH was injected sc at a dose of 50 ng in 0.2 ml of physiological saline.

At 21 days postoperatively, animals were weighed, killed by decapitation, and the pituitary gland weighed. The anterior pituitary was then separated from the posterior lobe under a stereomicroscope and weighed separately. Posterior pituitary weight was calculated as the difference between whole pituitay weight and anterior lobe weight.

Plasma and anterior lobes were stored frozen until assayed. Plasma and pituitary LH were measured by radioimmunoassay according to the method of Niswender *et al.* (6) and the results were expressed in terms of the NIH LH-S-1 ovine standard.[3] Significance of differences between means was determined by Student's *t* test or the paired *t* test as appropriate.

Results. Effects of lesions on plasma LH in castrated rats. Median eminence lesions uniformly lowered plasma LH in castrated males (Table I). Plasma LH was elevated in the castrated males with intact brains by a factor of 20 above that in intact males as expected, but lesions were associated with a

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[2] Supported by grants from NIH (AM 10073 and HD 05151) and the Ford Foundation.

[3] We are indebted to Dr. G. D. Niswender (Colorado State University) for the supplies of antiovine LH serum. Dr. L. Reichert (Emory University) generously provided the purified ovine LH for iodination.

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ISSN 0037-9727

o approximately 30% of the preop- ralues at 24 hr after the operation. LH remained low in these animals duration of the experiment. Sham- ns produced no effect on plasma istrated rats.

ffect of median eminence lesions on onse to LHRH in castrated males. iveness to LHRH was judged by ement in plasma LH at 10 and 20 owing injection of the neurohor- Table II). At 24 hr after lesions, is a marked increase in the size of ement in response to LHRH on son to that of control or sham-oper- which was apparent at both 10 and postinjection. The absolute concen- of LH in plasma following LHRH m the suppressed levels in the rats ions to values which were higher se found in the castrates with intact nd already elevated plasma LH; , the value for plasma LH concentration at 10 and 20 min after injection of LHRH was not significantly higher than that in the animals with intact brains in most experiments.

As previously reported (5), the increment in plasma LH following injection of LHRH was much greater in all castrate groups than in animals with intact testes (not shown).

By 7 days after lesions in castrates, there was a tendency for the responsiveness to LHRH to still be greater than that in sham-operated and intact castrates, but by this time, the increase was no longer significant. By 14 days after lesions, responsiveness was similar in castrate and intact animals whether or not they bore hypothalamic lesions.

Effect of median eminence lesions on body and pituitary weight, and anterior pituitary LH concentration. At sacrifice 1 week after the third LHRH response test, body weights of the animals in the various groups were similar (Table III). Median eminence le-

E I. PLASMA LH IN CASTRATE MALES BEFORE AND AFTER MEDIAN EMINENCE AND SHAM LESIONS.

Type of animal	Plasma LH (ng/ml ± SEM) time postlesion			
	Initial	24 hr	7 days	14 days
(4)[a]	19.83 ± 6.16[b]	24.56 ± 4.54	16.50 ± 3.77	12.50 ± 2.66
(4)	23.50 ± 1.93	22.00 ± 1.68	20.75 ± 2.25	22.25 ± 1.31
(4)	17.00 ± 1.99	21.00 ± 3.46	18.33 ± 3.20	25.26 ± 7.26
(4)	25.25 ± 7.79	20.00 ± 2.70	21.25 ± 3.42	32.00 ± 5.43
ninence lesions				
(8)	29.32 ± 4.17	8.24 ± 1.27*	8.30 ± 2.29*	5.15 ± 1.14*
(8)	27.50 ± 2.93	9.00 ± 1.30*	11.66 ± 2.06*	14.36 ± 3.68*

ber of rats.
± SEM.
ficantly lower than preoperative values ($P < 0.01$).

LE II. INCREMENT IN PLASMA LH FOLLOWING SC LHRH IN CASTRATE RATS WITH ME LESIONS.

mal	Increment in plasma LH (ng/ml ± SEM) time postinjections					
	1 day		7 days		14 days	
	10 min	20 min	10 min	20 min	10 min	20 min
	47.9 ± 10.0[b]	23.1 ± 9.3	39.3 ± 15.7	52.5 ± 27.5	75.0 ± 21.9	37.3 ± 4.8
	34.0 ± 4.3	22.8 ± 4.3	51.0 ± 3.3	32.5 ± 1.6	68.8 ± 10.5	57.7 ± 2.5
	51.1 ± 4.9	51.3 ± 14.5	44.4 ± 5.0	54.7 ± 3.4	70.8 ± 15.9	62.8 ± 11.6
	32.0 ± 4.8	10.6 ± 3.5	41.3 ± 7.1	28.3 ± 9.0	75.2 ± 5.1	42.3 ± 4.2
	78.7 ± 12.9*	71.8 ± 16.9*	73.0 ± 15.3	54.1 ± 14.3	59.7 ± 10.3	51.5 ± 8.8
	58.5 ± 9.0*	41.8 ± 10.0*	56.7 ± 7.7	37.8 ± 6.6	75.9 ± 9.5	67.9 ± 12.6

r of rats.
: SEM.
)5 vs control or sham.

sions were associated with a significant reduction in anterior pituitary weight on comparison to that of the control or sham-operated animals. Posterior pituitaries of the castrated animals with lesions were also smaller than those of the two control groups; however, this change was only significant in the case of the comparison of rats with lesions versus control castrates. Anterior pituitary LH concentration in the castrates with lesions was not significantly altered in one experiment, but in one experiment it was significantly elevated on comparison to that of sham-operated animals but almost the same as that of the intact controls. For unexplained reasons, pituitary LH values were higher in each group of the second experiment than comparable values in the first experiment.

Localization of median eminence lesions. Location of lesions was determined by stereomicroscopic inspection of the ventral surface of the brain at sacrifice and in some animals was verified by histological examination of serial sections through the lesions. These lesions were similar to those previously reported (2–4) in that they destroyed the anterior portion of the median eminence but left that portion just prior to the emergence of the pituitary stalk more or less intact. Thus, there was relatively little damage to hypophyseal portal vessels.

Discussion. The median eminence lesions in the present series were clearly effective in inhibiting LH release in castrates since they induced a sustained lowering of plasma LH which was apparent on first measurement at 24 hr after lesions and persisted for the 14-day duration of the experiment. The lesions were probably not complete, as evidenced by their failure to decrease pituitary LH concentration significantly in the castrates. There was a minimal decrease in size of the anterior pituitary as previously reported after median eminence lesions (1) and also a reduction in posterior pituitary weight presumably caused by interruption of the supraopticohypophyseal tract. Larger lesions would presumably have produced a greater decline in pituitary LH content as described earlier (1).

As previously reported, the response to LHRH determined by the increment in plasma LH at 10–20 min postinjection was greater in the castrates than in intact rats (5). This response was magnified further by median eminence lesions when the test was carried out 24 hr postlesions. Responsiveness had returned nearly to normal by 7 days and was in the normal range at 14 days after lesions. Clearly, responsiveness to LHRH is not impaired by lesions which inhibit the release of LH in castrate rats, which indicates that there is no obvious impairment in gonadotroph responsiveness caused by possible damage to the hypophyseal portal vasculature. In fact, responsiveness to LHRH was actually increased at 24 hr after these lesions.

Several possibilities suggest themselves as

TABLE III. BODY AND ORGAN WEIGHTS AND PITUITARY LH CONCENTRATIONS IN CASTRATE RATS WITH ME LESIONS.

Type of animal	Body weight	Post pituitary (mg/100 g body wt)	Anterior pituitary (mg/100 g body wt)	Anterior pituitary (mg total weight)	Anterior pituitary LH concentration (μg/mg of wet tissue)
Control					
Expt 1 (4)	407.7 ± 18.0	0.72 ± 0.12	3.34 ± 0.28	15.14 ± 1.06	38.35 ± 4.49
Expt 2 (4)	414.0 ± 15.3	0.53 ± 0.11	3.55 ± 0.20	16.36 ± 0.77	89.49 ± 2.58
Sham					
Expt 1 (4)	421.2 ± 9.9	0.90 ± 0.06	2.95 ± 0.26	13.82 ± 2.62	48.57 ± 7.47
Expt 2 (4)	387.0 ± 12.6	0.79 ± 0.30	3.62 ± 0.40	15.60 ± 2.18	73.92 ± 1.67
Median eminence lesions					
Expt 1 (8)	413.1 ± 21.6	0.47 ± 0.05*	2.61 ± 0.21*	12.0 ± 0.84*	33.38 ± 6.28
Expt 2 (8)	364.5 ± 22.5	0.43 ± 0.05*	2.76 ± 0.15*	11.2 ± 0.83*	90.13 ± 0.71**

* $P < 0.05$ versus control.
** $P < 0.05$ versus sham.

the cause for the increased responsiveness to LHRH in rats with median eminence lesions in the early postlesion period. The first possibility would be that diminished release of LH in the face of continued synthesis of the hormone may cause an increase in the size of a readily releaseable pool of LH which is then discharged following the injection of LHRH.

A second possibility is that in the absence of endogenous LHRH following lesions, the number of unoccupied LHRH receptors on the surface of the gonadotrophs is increased. Following the presentation of exogenous LHRH, there is an increased combination of the neurohormone with these receptors which leads to a greater discharge of LH.

The third possibility would be that following the loss of endogenous LHRH, the number of LHRH receptors on the cell surface increases. Following the injection of LHRH, an increased number of receptors combine with the neurohormone leading to increased LH release. The data provided in this paper cannot distinguish between these three possibilities to explain the increased responsiveness to LHRH at 24 hr postlesion.

The decline in responsiveness back to that observed in the sham-operated and control animals at 7 and 14 days after lesions may be related to a gradual suppression of LH synthesis in the presence of a chronic relative absence of LHRH. This would lead to a diminished releaseable pool of LH in the gland and thereby lead to releases of LH approximately the same as in animals with intact brains. Plasma LH was uniformly lowered after lesions and did not decline further as responsiveness to LHRH declined. This may be explained by increasing release of LHRH with partial recovery from the lesions which, when coupled with lessened responsiveness to LHRH, resulted in maintenance of subnormal plasma LH.

It should be pointed out that there are several examples of an apparent hypersensitivity to hormones following removal of the endogenous hormone. For example, following hypophysectomy, there is an immediate increase in responsiveness of the adrenal cortex to ACTH which is followed by diminished responsiveness later on (7).

Summary. Responsiveness to synthetic LHRH, as measured by increments in plasma LH following its sc injection, was evaluated in castrated rats with median eminence lesions and compared to that in sham-operated controls and controls with intact brains. Median eminence lesions in castrates were followed by a decline in plasma LH to approximately 30% of control values at 1, 7, and 14 days postlesions. This was associated with an increase in responsiveness to LHRH at 1 day, followed by a return to normal responsiveness at 7 and 14 days postlesions. Even though plasma LH was lowered in the animals with lesions, pituitary LH was little changed, which indicates that these lesions, although effective to inhibit LH release, were probably incomplete.

It is concluded that LH release is inhibited by lesions in the median eminence in the face of normal or even increased responsiveness to LHRH. The cause of the increased responsiveness to LHRH which appeared at 1 day after lesions remains to be elucidated.

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Received July 12, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 246-249 (1977)

Antibody Response to Enterobacterial Lipoprotein of Patients with Varied Infections Due to *Enterobacteriaceae*[1,2] (39647)

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The cell wall of members of the family *Enterobacteriaceae* is complex, both structurally and biochemically. It consists of lipopolysaccharide, nearest to the cell surface; protein and phospholipid of the outer membrane; and lipoprotein (LP) and peptidoglycan (murein) components of the innermost layer.

Lipoprotein is part of the rigid layer of the cell wall (1). The protein portion of the molecule consists of repetitive amino acid sequence (2) with its N-terminal end covalently linked to the lipid component (3) and its C-terminal end covalently bound to the murein layer (4). Approximately 250,000 lipoprotein molecules are linked to the murein (5). The murein-lipoprotein complex can be isolated in pure form after vigorous isolation procedures, such as treatment with 4% boiling dodecylsulfate, and lipoprotein can be further cleaved from the murein by treatment with trypsin (4, 6). When the protein is released from the murein, a morphological distortion of the wall occurs, which suggests that this protein plays an important structural role in the organization of the cell wall. Preliminary studies suggest that the lipoprotein chemically and antigenically is similar among the members of the family *Enterobacteriaceae* (5, 7).

In view of the possible biologic importance of antigens common to enterobacteriaceae, either for diagnostic purposes or for immunization, the present study was initiated to determine the antibody response to lipoprotein of patients with various enterobacterial infections. This investigation was also considered important because patients with these infections respond differently to another antigen common to enterobacteriaceae, the common enterobacterial antigen (CA) (8).

Subjects, materials, and methods. The following groups of patients from Children's Hospital and Roswell Park Memorial Institute, Buffalo, New York, were included in the study: (1) 33 patients with peritonitis, complicating appendicitis; (2) 33 patients with cystic fibrosis, complicated by enterobacterial respiratory tract infection; (3) 25 patients with enteritis, due to *Salmonella* or *Shigella;* (4) 25 patients with urinary tract infection, due to enteric gram-negative organisms; and (5) 33 patients with malignant disease, complicated by gram-negative bacteremia. In the first four groups, the age of the patients ranged from 1 month to 21 years, the majority from 1 to 14 years. The patients with malignant disease were all adults, ranging in age from 20 to 81 years. From these patients a total of 597 serum specimens were examined; from most subjects, at least 2 specimens and from many, multiple specimens were available for study. In addition, serum from 25 healthy children, from 7 to 14 years old, and 25 healthy adults were included for control purposes; the sera were kindly supplied by the Virology Laboratory at Children's Hospital.

Cultures were obtained from the appropriate sites of infection of all patients and microorganisms were isolated by conventional means. Antigens (O) from the patients' own microorganisms were prepared according to a previously described procedure (9, 10).

Two preparations of lipoprotein (LP) extracted from the cell wall of *E. coli* B were kindly made available by Dr. Volkmar Braun of the Universität Tübingen, Tübingen, Germany. One preparation was murein-lipoprotein, subjected to lysozyme degradation of the murein; the other was

[1] Dedicated to Felix Milgrom, M.D. on the occasion of 30 years of productive research.

[2] Supported by the Public Health Service Research Grant AI 00658, National Institute of Allergy and Infectious Diseases, USPHS.

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ISSN 0037-9727

tein released by trypsin from the mu-
ecause of availability, these studies
ırried out with the former prepara-
l the specificity of the antibodies was
:nted in selected sera with the latter
ıtion. The materials were received in
form and treated as follows. To 5.0
ipoprotein (LP) was added 0.5 ml of
NaOH; this mixture was incubated at
1 hr with occasional shaking. For
ization, 0.5 ml of 0.1 *N* HCl was
This stock solution contained 5000
of LP. Further dilutions were pre-
n phosphate hemagglutination buffer
(Difco; pH 7.3). All antigens were
at −20°.

n specimens were collected from pa-
ınd healthy subjects and stored at
rior to use. The passive hemaggluti-
test was used to titrate antibodies to
l to the O antigens of the infecting
rganisms. A 2.5% suspension of hu-
lood group O) erythrocytes was pre-
ınd washed three times in PHB. For
:ation with LP, either preparation in
:ntration of 50 μg/ml was added to
iment of the washed red blood cells
l a 2.5% concentration of the latter.
xture was incubated at 37° in a water-
)r 30 min. The erythrocytes were
vashed three times to remove unat-
antigen and resuspended to the origi-
centration. Modification of erythro-
'ith O antigen was accomplished by
cedure previously described in detail
. To titrate antibodies, serial twofold
ıs of serum in PHB were made in V-
wells of microtiter plates (Flow Lab-
:s, Rockville, Maryland), using mi-
crotiter diluters of 0.025 ml capacity
(Cooke Engineering, Alexandria, Virginia).
Then, 0.025 ml of PHB was added to each
well followed by 0.025 ml of antigen-modi-
fied erythrocytes. The plates were incubated
at 37° for 1 hr. Hemagglutination was deter-
mined by the settling pattern of the erythro-
cytes.

Results. The lipoprotein (LP) antibody
levels of healthy subjects and of patients
with varied enterobacterial infections were
determined. Whenever multiple specimens
from individual patients were available, the
highest titer was recorded. The results are
summarized in Table I.

Perusal of the table shows that none of
the healthy children and only one of the
healthy adults had antibodies against LP.
Nor did patients with salmonellosis, shigel-
losis, or urinary tract infection have detecta-
ble levels of these antibodies. In contrast,
18% of children with peritonitis complicat-
ing appendicitis, 24% of patients with enter-
obacterial infection of the respiratory tract
complicating cystic fibrosis, and 42% of
adult subjects with bacteremia complicating
malignancy, had LP antibodies in the serum.
Twelve of the 28 patients with LP antibod-
ies had elevated titers in the first available
serum specimen and no further increase was
observed in subsequent specimens. In con-
trast, an increase in the titer between acute
and convalescent specimens could be docu-
mented in 16 out of the 28 patients. Such an
increase occurred in 5 out of 6 subjects with
peritonitis and 10 out of 14 patients with
bacteremia. It is of interest to note that in
patients with cystic fibrosis, associated with
chronic infection, an increase in the titer of

E I. LP ANTIBODY RESPONSE OF VARIOUS GROUPS OF PATIENTS WITH ENTEROBACTERIAL INFECTION.

oups of subjects	Number of subjects	LP hemagglutinin titers[a] <2	2-8	16-64	Total number of subjects with LP antibody response
		Numbers of subjects (%)			
:hildren	25	25 (100%)	0	0	0 (0%)
ıdults	25	24 (96%)	1 (4%)	0	1 (4%)
s	33	27 (82%)	4 (12%)	2 (6%)	6 (18%)
ırosis	33	25 (76%)	3 (9%)	5 (15%)	8 (24%)
	25	25 (100%)	0	0	0 (0%)
ract infection	25	25 (100%)	0	0	0 (0%)
cy with bacteremia	33	19 (58%)	5 (15%)	9 (27%)	14 (42%)
	199	170 (85%)	13 (7%)	16 (8%)	29 (15%)

ıest titered serum specimen.

LP antibodies was documented in only 1 out of 8 cases; in the others, elevated titers did not change. These elevated titers persisted from 3 to 30 months. Analysis of the relationship between LP antibody response and age revealed that these antibodies were produced by children as well as adults, the youngest child being only 8 months old, the others ranging in age from 1 to 18 years. Of the 28 patients with a significant LP antibody level the enterobacteriaceae isolated included *E. coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter*. This observation provides suggestive evidence of the similarity of LP produced by various enteric bacteria, as proposed by Braun (5, 7).

Since these studies were carried out with the lysozyme-treated murein-lipoprotein preparation, which still retains fragments of murein at the C-terminal end of the protein, the specificity of the antibodies required clarification. To this end, 10 serum specimens containing antibodies against the antigen in elevated titers and 5 sera without these antibodies were tested in parallel with the murein-lipoprotein, as above, and with a preparation of lipoprotein cleaved from the murein by trypsin. In all instances the titrations yielded essentially identical results, the titers of the positive sera differing at most by one twofold dilution. Therefore, it is concluded that the antibodies demonstrated in the patients of this study were directed against the lipoprotein rather than against murein.

Finally, it was shown that an antibody response to the O antigens of the infecting microorganisms occurred more frequently than to the LP: 88% of patients with peritonitis, 88% of patients with enterobacterial infection complicating cystic fibrosis, 96% of patients with enteritis, 84% with urinary tract infection, and 85% of the patients with bacteremia complicating malignancy had a significant O antibody response.

Discussion. The lipoprotein (LP) portion of the cell wall of enterobacteriaceae, extensively studied by Braun and associates (4, 6), appears to be shared by various members of this family as revealed by analysis of the amino acid sequence (5) and immunologic properties (7). Such common antigens of microorganisms offer the opportunity of

family *Enterobacteriaceae* in the serum of healthy subjects and of patients with varied enterobacterial infections. LP antibodies were not demonstrated in the serum of healthy children and in only 4% of healthy adults. In contrast, 18% of patients with peritonitis complicating appendicitis, 24% of children with enterobacterial infection of the respiratory tract complicating cystic fibrosis, and 42% of patients with enterobacterial bacteremia complicating malignancy had these antibodies. LP antibodies were not detected in the serum of patients with *Salmonella* or *Shigella* enteritis or enterobacterial infection of the urinary tract. The specificity of the antibody was documented by the observation that with 10 serum specimens containing antibodies essentially identical titers were obtained with either the murein-lipoprotein or the lipoprotein preparation cleaved from murein as indicator antigens. The reason for these striking differences in the immune response to this common antigen remains to be elucidated.

The authors express appreciation to Dr. Volkmar Braun, Universität Tübingen, Tübingen, Germany, for his generous supply of lipoprotein.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 250-253 (1977)

Stimulation of Enzyme, Protein, and DNA Output from a Jejunal Fistula in the Rat (39648)

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We have modified the classical Thiry-Vella jejunal fistula, originally devised for dogs, for use in rats. This preparation has two main advantages over the use of dogs for studying the intestine: (i) The rat recovers rapidly from surgery and (ii) animal and maintenance costs are low. With this preparation we demonstrate the stimulatory effect of duodenal infusion of raw egg white on the output of DNA, protein, and enzyme activity from the fistula.

Methods. Surgical procedure. Food but not water is withheld for 16–18 hr before surgery. During the operation, maintenance of methoxyflurane anesthesia (Metafane, Pitman-Moore, Washington Crossing, New Jersey) is controlled by a vaporizing stream of O_2/CO_2 (95/5). A stainless steel rod with a small hole near one end is passed subcutaneously from a midline abdominal incision to a small dorsal incision through the skin on the rat's back. One end of both intestinal cannulae is passed through the hole in the rod and then pulled with the rod subcutaneously through to the abdominal incision. Intestinal cannulae are 25-cm lengths of Silastic medical grade tubing (0.64 × 1.19 mm) with a collar (1.02 × 2.16 mm) 1 cm from the beveled end (Fig. 1a). One cannula is inserted into the fistula and one into the duodenum. The free ends of the cannulae emerge through the skin dorsally out of the rat's reach. Sutures are placed at the small skin incision on the rat's back to secure the cannulae at their exit.

One centimeter above the ampulla of Vater a purse string suture is made with 5-0 suture and an intestinal cannula inserted through the gut wall and secured with ties above and below the collar. To form the fistula the gut is first transected between forceps about 2 cm distal to the ligament of Treitz and both cut ends are closed with an inverting suture and a second row of a purse string suture. About 0.5 cm from the closed end of the prospective fistula another intestinal cannula is inserted through the gut wall as described above for the duodenum (Fig. 1b). At the second transection, about 8 cm distal from the first, the distal end is closed and the proximal end is left open for isoperistaltic drainage of the fistula. Continuity of the gut is reestablished by side to side anastomosis. The open end of the fistula is brought out through an abdominal stab wound near the midline to protrude 0.5 cm from the skin. It is anchored by sutures to the peritoneum and subcutaneous fascia (Fig. 1c). A 0.5-cm segment of 1.0 × 0.80-cm Tygon tubing is fastened with Eastman 910 adhesive to the skin to surround the stoma of the fistula.

The animal is kept in a modified Bollman restraint cage. For the first 24 hr it has 5% dextrose solution to drink, after which time it has water and a powdered semisynthetic diet.[1] The rats eat readily and maintain their weight throughout the experimental period. The animals are usable after 2–3 days. In some fistulous animals, we have cannulated the jugular vein for infusion of drugs, and the common bile-pancreatic duct for collection of bile-pancreatic juice. These procedures were described in detail elsewhere (1, 2).

Experimental procedure. The fistula was perfused with saline at a rate of 10 ml/hr to clear the fistula of accumulated mucus and cellular debris. Samples were collected over ice for 30-min intervals. After 4 hr of basal perfusion, 350 mg of raw egg white (spray-dried, Nutritional Biochemical Co., Cleveland, Ohio) dissolved in 3 ml of saline, was

[1] Diet composition: Casein 24%, Jones and Foster salt mix, Nutritional Biochemical Co., 4%; vitamin mix, Nutritional Biochemical Co., 2%; corn oil, 6%; dextrin, 59%; Celluflour, 5%.

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ISSN 0037-9727

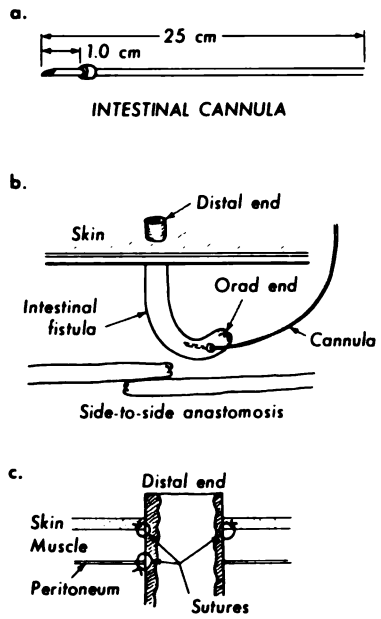

FIG. 1. (a) Cannula for insertion into intestine, or fistula, made of Silastic tubing. (b) Diagram of intestinal fistula and side-to-side anastomosis in the rat. (c) Details of suturing distal opening of fistula into the belly wall.

infused for a 25-min period into the duodenum or 3 ml of saline was infused as a control. The volume of the collected perfusate was measured, brought to 5 ml with distilled water, and homogenized in a ground-glass Duall tissue homogenizer with 12 up–down strokes. Three milliliters of the perfusate was treated for subsequent DNA analysis by the diphenylamine method of Goldsmith (3). The remaining homogenate was frozen and analyzed within 14 days for total protein (Lowry *et al.*, 4), peptidase activity (L-leucyl-β-napthylamide substrate, 5), and alkaline phosphatase activity (*o*-carboxyphenyl phosphate substrate, 6).

To test whether the mucosa of the fistula degenerated relative to the intestinal mucosa exposed to chyme during the time of the perfusion experiments, the isolated segment and two 10-cm intestinal segments orad and caudad from the anastomosis were removed 9–11 days postoperative. Each segment was rinsed three times with 2 ml of saline and slit longitudinally. The mucosae were examined macroscopically for any gross abnormality then scraped off with a glass slide, frozen on dry ice, lyophylized, and stored in a vacuum desiccator. The dried powder was ground with mortar and pestle and a weighed amount was homogenized in 10 ml of glass-distilled water for 1 min. The samples were kept at 4° during and after homogenization. The homogenates were analyzed as described previously for DNA, protein, peptidase activity, and alkaline phosphatase activity.

Results. As shown in Fig. 2, duodenal infusion of raw egg white, compared to saline infusion, increased the output of protein, DNA, peptidase activity, and alkaline phosphatase activity from an intestinal fistula ($P < 0.05$). The DNA output in the perfusate was threefold higher as a result of egg white infusion, which was the largest increase of the parameters measured. Protein, peptidase activity, and alkaline phosphatase activity were about 1.5-, 2.0-, and 2.5-fold higher, respectively ($N = 5$).

Table I summarizes the results of analyzing the mucosa from rats that were used in perfusion experiments. The protein and DNA content of the fistula mucosa was slightly greater than that of either adjacent intestinal segment ($P < 0.05$). Enzyme activity in the fistula was similar to that in the intestinal segment caudad to the anastomosis.

Discussion. The jejunal fistula described above allowed easy perfusion of the lumen and quantitative collection of the perfusate. Perfusion was facilitated by a small diameter cannula inserted into the orad end of the

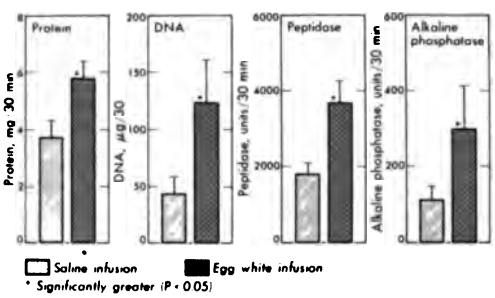

FIG. 2. Output of protein, DNA, peptidase, and alkaline phosphatase activity from a perfused (10 ml of saline/hr) intestinal fistula during infusion of saline or 350 mg of raw egg white into the duodenum in rats. Output of all parameters of secretion was significantly higher during egg white infusion ($P < 0.05$) ($n = 5$).

TABLE I. ANALYSIS OF MUCOSA FROM THE

	Intestine, ora anastom
Protein (mg)[b]	0.73 ± 0.
DNA (μg)[b]	39 ± 3.
Peptidase (units)[b]	408 ± 3
Alkaline phosphatase (units)	56.3 ± 1

[a] $N = 5$
[b] Each value is expressed as the amount per milligram
[c] Each value is mean ± SEM.

isolated segment (Fig. 1b). Infusion of substances into the intact duodenum was accomplished by a similar cannula. Cramer and Couch (7) developed a suturable stainless steel gastrointestinal cannula for use in fistulae in dogs, rabbits, and rats. These cannulae were inserted into each end of the gut segment and protruded through the belly wall; tubing was attached to the orad cannula for perfusion. We tried the method of Cramer and Couch on the distal end of a fistula with indifferent success. The cannula was awkward to secure, and the stainless steel exit tubing often became clogged with mucus. Simply allowing the distal end of the fistula to protrude 0.5 cm through the belly wall avoided clogging and prevented leakage of fistula contents into the abdominal cavity.

Animals were used for perfusion experiments for up to a week beginning on the 4th postoperative day after which they were killed for autopsy. The fistula often appeared slightly smaller in diameter than the intact intestine but the mucosal surface appeared macroscopically normal. Analysis of the mucosa showed a slight increase in the amount of protein and DNA per milligram of dried mucosa in the isolated segment (Table I). Several investigators have determined changes in morphology and function that occur in an isolated segment relative to the intact intestine (8, 9). After an intestinal resection, hypertrophic changes in the intact intestine near the anastomosis take place, but these changes are not significant until 4 weeks after operation (8, 9). At this time macroscopic atrophic changes and decreases in protein per centimeter of intestine and cell turnover time in the fistula are noticeable. Our rat preparation functioned normally for at least 1–2 weeks after operation.

species, including man. This work was before the announcement of pan-min activity by Harper and Raper Fink's fractionation procedures re- in a 300-fold concentration of entero- activity so that as little as 2 μg/kg iv in would give a measurable volume re- (16). Unfortunately, no other labo- has attempted to repeat the work on crinin and hence there is no other ce to confirm or confute the results referred to above. Several investiga- 17–19) have shown however, that ystokinin (CCK) stimulates secretion stinal enzyme activity. Protein in the ne stimulates the release of CCK from estine, as evidenced by an increased atic enzyme secretion (1). Therefore, nay have stimulated the positive intes- sponse to egg white. In humans CCK ates intestinal motor activity (20). In- d motility could increase desquama- te in the intestine which would be ed in the increased enzyme and DNA

mary. A new technique for preparing nal fistulae in rats is presented. Perfu- f the fistula is facilitated by inserting liameter tubing into the closed orad the fistula. The distal end protrudes a through the belly wall for quantita- ollection of perfusate or secretion. uity of the gut is reestablished by a -side anastomosis. Analysis of the a from the fistula and from the intact ne demonstrated that the mucosae are rable during the week the animals are or perfusion experiments. The results monstrate that duodenal infusion of g white stimulates the output of pro- DNA, peptidase and alkaline phos- e activity in the jejunal fistula per-

This work was supported in part by Postdoctoral Fellowship 5 F22 AM01909 and Grant AM 15258 from the National Institutes of Health, DHEW.

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Received September 7, 1976. P.S.E.B.M., 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 254-258 (1977)

TRH-Induced Growth Hormone (GH) Release in Rats of Both Sexes: Changes in Pituitary Response after Gonadectomy and During the Estrous Cycle[1] (39649)

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Thyrotropin-releasing hormone (TRH) is known to stimulate at high doses the release of pituitary GH both *in vivo* and *in vitro* (1-4). TRH can also stimulate GH secretion in patients with anorexia nervosa, renal failure, mental depression or acromegaly and in lactating cows (5-9).

To determine if the gonads might influence the pituitary GH response to TRH, the neurohormone was injected intravenously into ovariectomized or intact female rats at different phases of the estrous cycle and in intact or castrated male rats and its effect on pituitary GH release was evaluated by measuring radioimmunoassayable plasma GH levels following the injection.

Materials and methods. Young adult male and female rats (55-60 days of age) of the Holtzman strain (Holtzman Co., Madison, Wisconsin) were used. They were housed under controlled conditions of lighting (14 hr on, 10 hr off) and temperature (24-26°). Tap water and Purina laboratory chow were available *ad libitum*.

Gonadectomy. Males were castrated 6 days before TRH injection through a single midline incision in the scrotum. Female rats were castrated at day 22-23 of age via the dorsal approach. Ether anesthesia was used for all operative procedures.

TRH injections. Animals were anesthetized with tribromoethanol (10) at a dose of 25 mg/100 g body wt administered ip 60 min before injecting the TRH. Maintenance doses of the anesthetic (1/2 the initial dose) were administered sc 45 and 5 min before TRH injection. The tripeptide (Beckman) was dissolved in 0.9% NaCl solution at a concentration of 8.5 μg/ml and injected iv at a dose of 850 ng/100 g body wt. Control animals were injected with saline solution (0.1 ml/100 g body wt). Blood samples were drawn from the jugular vein into heparinized syringes immediately before or at 2, 5, and 10 min following the injection of TRH or the diluent. When cyclic females were used, vaginal smears were obtained daily and only those rats exhibiting at least two consecutive 4-day cycles were injected with TRH. All experiments were performed in the morning.

Pituitary GH measurements. In another experiment, different groups of animals were decapitated, their pituitaries dissected out, the neurohypophysis removed and the anterior lobe placed on dry ice. Thereafter, the glands were weighed to the nearest 0.1 mg and homogenized in cold 0.9% NaCl. Following low speed centrifugation, the supernatants were separated and stored at −20° until assayed. When pituitary GH of intact female rats was determined, the animals were classified according to the vaginal cytology they presented on the day of sacrifice. All animals were killed in the morning.

Radioimmunoassay. Plasma and pituitary GH were measured by radioimmunoassay using a kit supplied by the NIAMDD.[3] To avoid interassay variation, samples from a complete experiment were assayed in the same assay. Intra-assay variation was 9.5%. Interassay variation was 12.7%. Results are expressed in terms of the NIAMDD rat GH-RP-1 standard supplied with the kit.

Statistics. Significance of differences between means of two groups was determined by Student's *t* test. Differences between pre-

[1] Supported by grants from NIH (AM 10073 and HD 05151), the Ford Foundation, and the World Health Organization.

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[3] The kit for determination of GH was provided through the NIAMDD-NIH Pituitary Hormone Program.

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ISSN 0037-9727

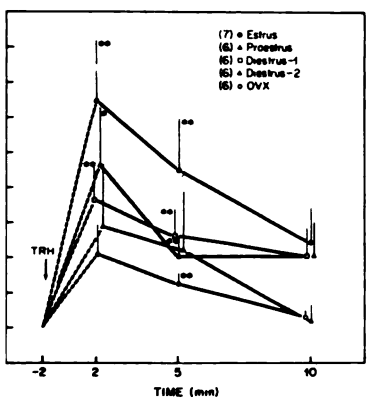
tinjection hormone values in the ıup were analyzed with the paired *t*

ɾ. *Initial plasma GH levels.* One ɛr initiation of tribromoethanol an- plasma GH levels were signifi- gher ($P < 0.005$) in intact male rats 25 ng/ml) than in castrated males 9.1 ng/ml) or than those in intact ɛrated females ($P < 0.025$–$P <$ Although GH levels of estrous fe- 2 ± 10 ng/ml) tended to be greater se of females in proestrus (47 ± 5 liestrus I (48 ± 8 ng/ml), diestrus II) ng/ml), or in ovariectomized ani- ± 4 ng/ml), these differences were ɜtically significant.

of TRH on GH release in female ɜ injection of TRH into intact fe- s induced a significant increase in ɜH at all phases of the estrous cycle exception of diestrus day 2 (Fig. 1). mean responses were observed in at 2 min following the injection. ɦ the GH release induced by TRH and 10 min after its injection was n estrus than at the other phases of , these differences were not statisti- ıificant. Long term ovariectomy (at lecreased the GH response to TRH y-old rats, the increase in plasma GH being significant only at 5 min ($P <$ 0.025).

Effect of TRH on GH release in male rats. Intact male rats injected with TRH showed an increase in plasma GH levels greater than that of females injected with the neurohormone at any stage of the cycle (Fig. 2). GH titers were maximal at 2 min ($P < 0.01$) and decreased rapidly thereafter, although they were still significantly elevated at 5 min ($P < 0.05$). When TRH was injected in castrated male rats, the GH response to the tripeptide was significantly ($P < 0.005$) blunted. The small increase in plasma GH observed after TRH in these animals was significant only at 2 min ($P < 0.05$).

Maximal increment in plasma GH levels induced by TRH in male and female rats. The maximal increase in GH levels following TRH was not always attained at the same time in animals of the same group. Therefore, the maximal GH increment induced by TRH in each animal was determined and the mean values obtained for the different groups were compared. Figure 3 illustrates these results. Ovariectomized rats released less GH than rats in estrus ($P <$ 0.05) but not significantly less than animals in proestrus, diestrus day 1 or diestrus day



Growth hormone release induced by TRH 00 g body wt) at different phases of the ɛle and in ovariectomized rats. * = $P < 0.05$ ɛct to initial value, ** = $P < 0.025$ with initial value. In this and subsequent figures, es represent standard error of the mean and ı parentheses next to the key indicate num- nals used.

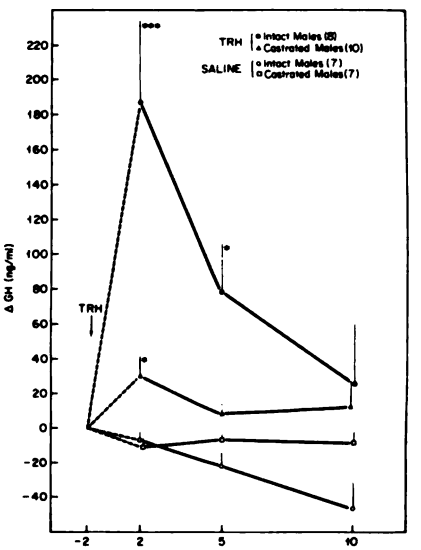


FIG. 2. Effect of gonadectomy on the GH release induced by TRH injection (850 ng/100 g body wt) in male rats. * = $P < 0.05$ with respect to initial value, *** = $P < 0.01$ with respect to initial value.

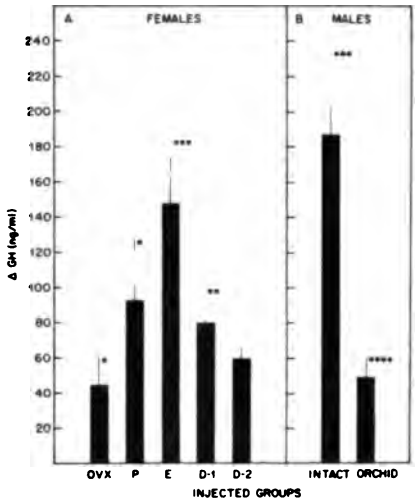


FIG. 3. Comparison of the effect of TRH (850 ng/100 g body wt) on GH release in intact or castrated female and male rats. The figure depicts the maximal increment in GH observed at any of the postinjection times studied (2, 5, and 10 min). Abbreviations: OVX, ovariectomized; P, proestrus; E, estrus; D-1, diestrus day 1; D-2, diestrus day 2; Orchid, orchidectomized. **** = $P < 0.005$. For other statistical significances, see Figs. 1 and 2.

2. The GH response to TRH was greater ($P < 0.05$) in estrus than in diestrus day 2.

Intact male rats released much more GH in response to TRH than castrated animals ($P < 0.01$). In spite of the dramatic decrease in GH response after castration, TRH was still able to induce a significant ($P < 0.005$) release in the castrated animals.

Pituitary GH in intact or castrated male and female rats. Pituitary GH content and concentration tended to be higher in intact females in diestrus than in animals in estrus (Table I). However, this difference was not statistically significant. Long-term ovariectomy resulted in a significant increase in both content ($P < 0.01$) and concentration ($P < 0.005$) of GH in comparison to the GH content in female rats in estrus. Pituitaries of male rats had significantly greater GH content ($P < 0.005$) and concentration ($P < 0.001$) than intact female rats. Orchidectomy resulted in a decrease in both content ($P < 0.005$) and concentration ($P < 0.01$) of GH in the gland.

Discussion. The present results show that GH release induced by TRH is facilitated by the presence of the gonads in both male and female rats and that in the female, cyclic variations in ovarian activity which occur during the estrous cycle of the animal also result in changes in pituitary GH responsiveness to the tripeptide. Although it is conceivable that the anesthetic procedure used may have influenced the results, other authors (4) have demonstrated that the GH response to TRH injection is not different between animals maintained constantly anesthetized or animals that were briefly etherized during the collection of blood samples. We have recently found that the pattern of GH release induced by TRH in conscious, free moving female rats is essentially the same as that found in tribromoethanol anesthetized rats.

That TRH acts on the pituitary to induce GH release has been convincingly demonstrated by the observations that TRH can induce GH release in rats with extensive hypothalamic ablation (4) when infused into the portal vessels (2) or *in vitro* from perifused rat hemipituitaries (3).

Although after column chromatography of hypothalamic extracts, GH-releasing factor (GH-RF) was found to have an elution volume similar to that of TRH (11–13), TRH alone could not account for the whole GH-releasing activity of the purified hypothalamic extract (12). This and other observations (1–3) have led to the suggestion (3,

TABLE I. PITUITARY GH CONTENT AND CONCENTRATION OF ADULT (55-56 DAYS OF AGE) INTACT AND CASTRATED MALE AND FEMALE RATS.

Groups	No. of rats	GH content (μg/pit)	GH concentration (μg/mg)
Intact females (estrus)	16	365 ± 27[1]	43.9 ± 3
Intact females (diestrus)	12	427 ± 37	54.3 ± 5
Castrated[2] females	4	542 ± 35[a]	70.5 ± 11.8[b]
Intact males	9	632 ± 41[c]	86.9 ± 6.7[c]
Castrated[3] males	7	381 ± 58[d]	56.9 ± 7.5[e]

[1] Mean ± SEM
[2] Ovariectomized at day 22–23 of age.
[3] Orchidectomized 6 days before decapitation.
[a] $P < 0.01$ vs estrus.
[b] $P < 0.005$ vs estrus.
[c] $P < 0.005$ vs females in diestrous phase.
[d] $P < 0.005$ vs intact males.
[e] $P < 0.001$ vs intact males.

t TRH and GH-RF may share certain
ion structural features which can ac-
for their stimulatory action on GH
e.

: diminished GH response to TRH fol-
g gonadectomy is in all likelihood due
noval of gonadal steroids. A facilita-
ffect of sex steroids on basal growth
one release has been suggested earlier
6), and, in fact, estrogen treatment
een shown to increase circulating GH
in female rats (17). Malacara and
lin (13) previously reported that treat-
with estrogen and progesterone sensi-
the pituitary gland to the GH-releasing
of porcine hypothalamic extracts. In
:r support of the concept of a modula-
ıction of gonadal steroids at the pitui-
evel on GH secretion are the observa-
that following gonadectomy there
marked changes in pituitary GH con-
nd concentration. Interestingly, pitui-
GH increased after ovariectomy and
ased after orchidectomy, a finding that
ms the earlier report of Birge *et al.*
who also showed that testosterone
nent resulted in an increase in pituitary
ontent and concentration, whereas es-
n led to a decrease in pituitary concen-
n of the hormone. It is unlikely that
line extraction of pituitary GH used in
present experiments influenced the
ırement of GH because the results
in complete agreement with those of
et al. (18) who homogenized the
s in 0.01 *N* NaOH. Moreover, these
rs found that the mean yield of GH in
genates of NaOH, acetic acid or phos-
line buffer as measured by RIA did
gnificantly differ between the different
s.

hough the administration of both es-
n and androgens enhances the GH re-
e to provocative stimuli (14–16, 19),
echanism by which these steroids exert
effect on GH release is probably differ-
nce they have opposite effects on pitui-
GH stores. The greater GH responsive-
of male than female rats to TRH may
ated to the fact that pituitary GH con-
nd concentration in males is greater
n females. Interestingly enough, in the
n, the GH response to provocative stimuli is clearly greater in females than in males (14–16, 19). In the rat, the existence of a sex difference in GH content was suggested by Jones *et al.* (20) and clearly established with the use of radioimmunoassay by Birge *et al.* (18). Lower pituitary GH values in female rats are probably related to an increase release of the hormone induced by circulating estrogen levels (17) without a concomitant increase in the rate of synthesis. Higher basal plasma GH levels in female than in male ambulatory patients have been reported (21) as well as an increase in plasma GH levels during the preovulatory period (22), a time at which estradiol levels also rise (23).

Dickerman *et al.* (17) reported that plasma GH levels were higher in estrus than at other phases of the cycle. The present finding that GH responsiveness to TRH is also more pronounced in estrus suggests that an increased GH response to GH-RF and/or TRH can at least in part account for the elevated GH levels observed at that phase of the cycle.

The fact that TRH can induce GH release and that the magnitude of this response is modified by altered thyroid (24) or gonadal status raises the possibility that TRH can be used as a tool to study the capacity of the pituitary gland, to release GH under varied physiological or pathological conditions.

Summary. In both intact adult male and female rats anesthetized with tribromoethanol, the iv injection of thyrotropin-releasing hormone (TRH) evoked GH release within 2 min following the injection. The GH response to TRH was greater in males than in females and this response was significantly diminished by short-term (6 days) orchidectomy. In females, the GH response to TRH was maximal in estrus and minimal in diestrus day 2, showing intermediate values at proestrus and diestrus day 1. Long-term ovariectomy decreased the response. Pituitary GH content and concentration were higher in males than in females. Orchidectomy was followed by a significant decrease in pituitary GH values, whereas ovariectomy resulted in increased GH content and concentration. The results indicate that the gonads can modify pituitary GH response to TRH and, therefore, play a physi-

ological role in modulating the stimulatory effect that the hypothalamus exerts on GH release through the secretion of substances with GH-releasing activity.

We appreciate the technical assistance of Miss Helen Jameson. We also thank Miss Cheri Reimann for secretarial assistance.

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Received September 20, 1976. P.S.E.B.M. 197 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 259-263 (1977)

Lysis of Cytomegalovirus-Infected Human Fibroblasts and Transformed Human Cells by Peripheral Blood Lymphoid Cells from Normal Human Donors (39650)[1]

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The cellular immune response to cytomegalovirus (CMV) infection in man has not been well defined. However, the importance of cellular defenses is suggested by the association of severe CMV infections with immune-deficiency states and immunosuppression (1).

Several studies have delineated various cellular cytotoxic mechanisms capable of the destruction of cells infected by other members of the *Herpes* virus group. For example, antibody-dependent cell-mediated cytotoxicity (ADC) has been implicated in the immune elimination of *Herpes simplex* virus (HSV)-infected cells (2, 3). Hutt *et al.* (4) demonstrated the cytotoxicity of normal human leukocytes for Epstein-Barr virus (EBV)-transformed human lymphoblastoid cell lines which was increased in patients convalescing from infectious mononucleosis. Our experiments were undertaken to evaluate the possible role of various cell-mediated cytotoxic mechanisms in the destruction of target cells infected with CMV.

In this study, using human peripheral blood mononuclear cells from normal nonimmune donors, we observed the unexpected lysis of CMV-infected human fibroblasts. The observed lysis of CMV-infected human fibroblasts appeared to resemble descriptions of "spontaneous" cytolysis of several cell lines by nonimmune lymphocytes (5-9). Therefore, lysis of CMV-infected fibroblasts is compared with activity against three "transformed" human cell lines.

Materials and methods. Human neonatal foreskin fibroblast (FS) cell cultures and stocks of CMV strain AD-169 were prepared as previously described (10). During the course of the experiments, four different preparations of FS cell cultures were used. CMV-infected FS cells were prepared in two manners. Monolayers of FS were infected with trypsin-dispersed infected cells (at a 1:3 ratio of infected: noninfected cells) and used 3 to 6 days later when infection was nearly complete. For infection with free virus, 24-hr-old FS cells were infected at a multiplicity of infection of 1 and utilized 24 to 72 hr later. Uninfected FS cells were grown in a similar manner. These, as well as the HEp-2 and Chang cell lines, were grown in Eagle's Minimal Essential Medium supplemented with 1% glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of amphotericin B, and 10% fetal calf serum. The omission of amphotericin B in some experiments did not alter the observed cytolysis. Suspension cultures of the P2HR-1 cells (11), an EBV-transformed lymphoid cell line, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Monolayer cultures of target cells were suspended by incubation with 0.05% trypsin in 0.05% EDTA for 5 min. All futher cell incubations were performed in RPMI 1640 medium (Hepes-buffered) supplemented with 10 m*M* L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% agamma or normal fetal calf serum. Viable target cells (5×10^6/ml) in 1 ml of medium were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 hr at 37°. After washing four times, target cells were counted using trypan blue, and 5×10^4 target cells were dispensed into tubes in a 0.1-ml volume. The incubation tubes were flat-bottomed 10 ×

[1] Supported by N.I.H. General Research Support Grant 5476 from the Division of Research Resources, and the Medical Research Institute Council, Michael Reese Hospital.

[2] Recipient of Research Career Development Award 00055 from N.I.A.I.D.

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ISSN 0037-9727

47-mm glass (Becton Dickinson, Rutherford, New Jersey), which had been washed with deionized water and autoclaved before use. Tubes with labeled target cells were incubated at 37° for 3 hr to permit formation of a stable monolayer.

Human peripheral blood was obtained from 14 normal donors with complement fixation (CF) titers of less than 1:8 to CMV and one patient convalescing from CMV mononucleosis (documented by a fourfold CF titer rise). To verify further the immunologic status of donors, sera were tested by the more sensitive (12, 13) indirect fluorescent antibody (IFA) technique (10). This was modified from our earlier procedures by using 24-hr-old CMV-infected fibroblasts to minimize confusion of anti-viral nuclear antibodies with cytoplasmic IgG-Fc receptors, which become maximal at 72 hr after infection. Of 11 donors tested by IFA, only one was positive, at a 1:4 dilution. Fresh cord blood was obtained after normal deliveries. Most blood was defibrinated with glass beads, but some samples were heparinized with no discernible effect on results. Mononuclear cells were separated using Hypaque-Ficoll as outlined by Böyum (14). In some cases, phagocytic cells were eliminated by modifications of the techniques of Lundgren *et al.* (15). Blood was mixed in a siliconized flask with 1 mg/ml of carbonyl iron (GAF, Manchester, England) which had been sonicated just before use. Flasks were incubated on a rocking platform for 60 min at 37° and were swirled by hand every 5 min to ensure further, mixing. After repeated passages through a magnetic field, the remaining lymphocytes were separated by Hypaque-Ficoll. After neutral red or latex particle ingestion, only 0.5–1.5% phagocytic cells remained. T-cell enriched populations were prepared according to the technique of Julius *et al.* (16). After Hypaque-Ficoll separation, mononuclear cells were diluted in MEM containing 5% fetal calf serum and incubated at 37° for 45 min in columns containing washed, sterile, nylon wool (LP-1 Leuko-Pak Leukocyte Filters, Fenwall Laboratories, Morton Grove, Ill.). Effluent cells were washed three times before use.

Leukocytes were suspended in RPMI 1640 (Hepes-buffered and supplemented as outlined above) with 5% fetal calf serum and added to ^{51}Cr-labeled target cells. The final volume in each tube was adjusted to 0.8 ml with medium. After an 18-hr incubation at 37°, 0.4 ml of the supernate was carefully removed from each tube, and counted in a gamma counter. The total releasable radioactivity from each group of target cells was determined using specimens lysed with 1% Triton X-100. Spontaneous ^{51}Cr release was determined from tubes containing no effector cells. The percentage of specific lysis was computed as: (mean ^{51}Cr release in test group − mean spontaneous ^{51}Cr release)/(mean ^{51}Cr release after total lysis − mean spontaneous ^{51}Cr release) × 100. All experiments were performed in triplicate.

Results. In initial experiments using leukocytes from 14 normal donors. the maximum specific lysis of 32.3 ± 4.2% (mean ± SE) CMV-infected FS cells was observed. Maximum cytolysis occurred at effector:target cell ratios of 100–400:1. However, at the target cell numbers (5×10^3 cells/tube) used in these experiments, it was found that uninfected FS cells spontaneously released unacceptably high percentages of ^{51}Cr. Using a 10-fold greater density of target cells (5×10^4/tube), both CMV-infected and uninfected FS cells proved more stable; spontaneous ^{51}Cr release, in the absence of effector leukocytes, was 15.9–32.7% after 18 hr.

Using this greater target cell density, CMV-infected FS cells were lysed by leukocytes from normal adults, whereas uninfected fibroblasts were not (Table I). Cytolysis of CMV-infected FS cells was proportional to the number of effector cells present at effector:target cell ratios of 6.25–50:1, but declined at higher ratios. An examination of the time course for the development of susceptibility to cytotoxicity after CMV infection demonstrated lysis as early as 24 hr postinfection. Similar levels of lysis were observed in 24- and 72-hr-old infected FS cells (50.1 and 58.2%, respectively).

In order to determine if the observed cytotoxicity is present from birth, lysis of CMV-infected FS cells by cord blood mononuclear leukocytes was studied. It was found

TABLE I. CYTOLYSIS OF CMV-INFECTED AND UNINFECTED FIBROBLASTS BY LEUKOCYTES.

et cell	Source of leukocytes (number tested)	Specific lysis at the following ratios of leukocytes:target cells[a]			
		50:1	25:1	12.5:1	6.25:1
:s	Normal subjects (14)	2.6 ± 0.8	4.8 ± 0.9	4.6 ± 1.4	5.9 ± 2.3
:cted fibro-	Normal subjects (14)	53.8 ± 3.9	42.3 ± 4.4	21.6 ± 3.8	11.8 ± 2.1
:cted fibro-	Cord blood (3)	6.7 ± 1.7	8.9 ± 3.2	6.9 ± 4.8	4.3 ± 1.5

:ntage of ^{51}Cr release ± SE.

rd blood leukocytes induced only lysis of CMV-infected fibroblasts). Since active CMV infection might :ted to enhance cellular immunity to fected target cells, we studied one :onvalescing from CMV mononucle- the time of study (4 weeks after his leukocytes lysed CMV-infected he mean rate of leukocytes for nor- jects.

ıse of the known importance of destruction of HSV-infected cells, ied the role of specific antibody in :tion. Anti-CMV antibody (1:10 di- of two sera from convalescent pa- ith 1:320 and 1:1024 CF titers) did gment cytotoxicity. In contrast, ell lysis was markedly augmented by nti-Chang cell antibodies (two- to d increased lysis at effector:target os of 25–50:1, as well as significant y-dependent lysis at ratios as low as Moreover, we noted no reduction in ls of cytotoxicity of CMV-infected en the reactions were carried out in ence of specific viral antibody. Be- MV infection induces the develop- an IgG-Fc receptor in FS cells (10), lied the possibility that γ-globulin in fetal calf serum might mediate n eight experiments using agamma f serum substituted for fetal calf se- o difference in cytotoxicity was

ocytes were further separated in or- tudy the nature of the effector cell ı the spontaneous lysis of CMV-in- target cells. In two experiments, nt of Hypaque–Ficoll-separated leu- with carbonyl iron and a magnet to phagocytic cells did not change cy- y. Hypaque–Ficoll-separated leuko- cytes were then passed through a nylon wool column. This almost totally depleted immunoglobulin-bearing cells (determined by direct immunofluorescence using a polyvalent goat anti-human immunoglobulin serum). In two experiments, this resulted in 52 and 60% decreases in cytotoxicity compared with Hypaque–Ficoll-separated leukocytes.

The lymphocyte-mediated spontaneous lysis of the CMV-infected FS cells observed in these studies appeared to resemble descriptions of lysis of transformed cell lines. Therefore, further studies were done to compare the lysis of different cell lines by leukocytes from normal donors. In general, the lysis of CMV-infected FS cells was in the same range as the lysis of three transformed human cell lines, i.e., HEp-2, Chang, and P3HR-1 (Table II).

Discussion. The results presented here demonstrate that human peripheral blood leukocytes from nonimmune donors are capable of injuring CMV-infected human fibroblasts. No such cytotoxicity was observed when similar effector cells were combined with uninfected target cells. Effector cells from all 14 normal donors tested demonstrated this nonspecific cytotoxicity. Moreover, the cytotoxic effect did not appear to be mediated by T cells, and it occurred independent of the presence of specific anti-viral antibodies. These findings suggest that this activity is not directed against the viral antigens displayed on the surface of CM-infected cells. Rather, it may be directed against some modification of the cell surface induced as a result of the virus infection.

Recent findings from this laboratory (10) and others (17) have shown that CMV infection induces the formation of an IgG-Fc receptor in human cells. This IgG-Fc recep-

TABLE II. COMPARISON OF CYTOLYSIS OF CMV-INFECTED FIBROBLASTS WITH CYTOLYSIS OF HUMAN CELL LINES BY LEUKOCYTES FROM NORMAL SUBJECTS.

Target cell (number of experiments)	Specific lysis at the following ratios of leukocytes:target cells[a]			
	50:1	25:1	12.5:1	6.25:1
CMV-infected fibroblasts (14)	53.8 ± 3.9	42.3 ± 4.4	21.6 ± 3.8	11.8 ± 2.1
HEp-2 (14)	41.2 ± 4.5	26.3 ± 5.4	16.9 ± 5.4	2.1 ± 5.9
Chang (2)	42.5	29.2	11.5	2.9
P3HRI, EBV-transformed (1)	45.2	41.3	29.9	15.9

[a] Percentage of ^{51}Cr release ± SE.

tor is present on cell surfaces and has been detected in tissue culture fluids as early as 24 hr postinfection (18). If IgG aggregates or immune complexes are present and react with IgG-Fc receptors on CMV-infected cells, they might then be expected to react with the IgG-Fc receptors of ADC cells. Therefore, one possible mechanism for this apparent natural cytotoxicity may be through the linking of ADC effector cells to virus-infected target cells. In fact, such a mechanism has recently been proposed to account for the natural cytotoxic effect demonstrated by normal murine macrophages against HSV-infected mouse neuroblastoma cells (19). However, the mediation of ADC by IgG complexes in our system would seem unlikely, based on our findings of equal levels of cytotoxicity when experiments were conducted in the presence or absence of IgG, i.e., agamma calf serum. Nevertheless, this possibility has not been totally excluded since the effector cell population contained B cells which conceivably, might have produced IgG. The hypothetical role of IgG microaggregates and ADC effector cells in this system would be consistent with our finding that nylon wool column-purified lymphocyte populations depleted of B lymphocytes demonstrated a 50 to 60% decrease in cytotoxic activity. We are currently studying ADC as a possible mechanism for natural cytotoxicity in this system.

The cytotoxicity developed by human leukocytes against CMV-infected cells appears to be similar to the nonspecific cytotoxicity observed with the three transformed human cell lines examined. On the basis of the present data, we are unable to conclude that the surface signal produced by the CMV infection is similar to that present on human transformed cell lines. However, it is of interest to note that CMV-infected cells demonstrate some alterations of the cell surface which are characteristic of transformed cells. Thus, although uninfected human fibroblasts are unable to replicate in media containing agarose, CMV-infected human diploid fibroblasts acquire the ability to grow into small colonies in the presence of agarose (20). Furthermore, human fibroblasts infected with CMV assume an epithelioid conformation and continue active division during the first few days following infection, prior to undergoing cellular lysis (personal observations). CMV infection stimulates host cell DNA synthesis, an important characteristic shared with oncogenic DNA viruses (21). Indeed, CMV has been shown capable of inducing oncogenic transformation of hamster embryo fibroblasts (22) and human embryo lung cells (23). On this basis, it is tempting to speculate that the natural cytolysis of CMV-infected cells is directed against an early alteration of cell membranes common to several types of human transformed cells.

Two serologic parameters failed to detect evidence of previous immunologic experience with CMV in all but one normal donor. Nevertheless, lymphocytes from that donor and one CMV convalescent donor did not demonstrate greater cytotoxicity against CMV-infected fibroblasts than cells from nonimmune donors. Cytolysis of CMV-infected fibroblasts closely paralleled the lysis of three transformed cell lines. On this basis, the ineffectiveness of peripheral blood leukocytes obtained from cord blood to serve as cytotoxic effector cells suggests the involvement of an immunologic response to altered cell surface antigens rather than a direct response to viral antigens. Alternatively, this may be explained by the

unctional prematurity of the effector cell population responsible for this natural cytotoxicity. However, it should be noted that these results differ from results reported by Pross and Jondal (6) who noted the cytolysis of human cell lines by cord blood lymphoid cells, though their data is not reported in detail.

The biological significance of spontaneous cytotoxicity is unclear. Nevertheless, the failure of leukocytes to lyse normal fibroblasts in our studies suggests a possible role for spontaneous cytotoxicity in normal surveillance against virus-infected and transformed cells in man.

Summary. Peripheral blood leukocytes from 14 healthy, nonimmune, human donors were capable of destroying CMV-infected human fibroblasts, but non uninfected fibroblasts. Antibody did not appear to mediate the response. Cytotoxicity in a similar range was noted when three transformed human cell lines were used as target cells. Unlike adult leukocytes, leukocytes separated from three samples of cord blood were ineffective in destroying CMV-infected target cells.

We thank Dr. Roberto Kretschmer for providing cord blood and Ricki Peitchel for technical assistance.

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Received July 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 264-268 (1977)

Mouse Thymocyte Membrane Uptake of Polyadenylic-Polyuridylic Acid Complexes (39651)

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Complexes of homoribopolymers such as polyadenylic acid and polyuridylic acid [poly(A:U)] are known stimulators of thymocyte activity in both cell-mediated immunity and humoral antibody formation (1). Although the mechanism of action is unknown, it has been suggested that the effects of poly (A:U) are mediated through the cell membrane (2). However, Fenster *et al.* (3) have shown that poly (A:U) rapidly penetrates certain mammalian cells and is soon located in the cell nuclei. Consequently, we have studied the uptake of poly (A:U) *in vitro* by mouse thymocytes utilizing indirect immunofluorescence. Evidence is presented which indicates that approximately 80% of thymocytes bind poly (A:U) on their membranes.

Materials and methods. Animals. Three-month-old New Zealand white male rabbits were used for formation of antibody to poly (A:U). Balb/Aj male and female mice inbred in our laboratory were used throughout.

Preparation of antisera. Poly (A) (Lot No. 77 Code 11-301), poly (U) (lot No. 72 Code 11-308), and methylated bovine serum albumin (MBSA lot 15) were obtained from Miles Laboratories, Inc., Elkhart, Ind. Complete Freund's adjuvant was obtained from Difco, Co., Detroit, Michigan. The polymers were prepared at a concentration of 5 mg/ml in 0.15 *M* NaCl and mixed to form the helical polymer, poly (A:U). These solutions were refrigerated at 4°.

Dilutions were made in 0.15 *M* NaCl to a concentration of 1 mg/ml. MBSA was prepared in a 1% aqueous solution. Equal volumes of the two solutions were mixed, and the complex was emulsified with an equal volume of Freund's complete adjuvant to a final concentration of 454 μg of antigen/ml. Fresh antigen was prepared at the time of each injection.

Rabbits were injected intramuscularly with 1.4 ml of the antigen preparation in divided doses, weekly for 4 weeks. The final injection, administered in the fifth week, did not contain Freund's adjuvant. The animals were bled 8 days following the last injection.

Sera were heat inactivated and absorbed four times using BALB/Aj spleen and thymocyte cells at a ratio of 1 vol of cells to 3 vol of sera. The sera were filtered and diluted 1:16 with 0.01 *M* PBS, pH 7.2, and frozen at −20°.

Ouchterlony double diffusion was used to evaluate the specificity of the antisera to poly (A:U). Precipitation was found at all concentrations of poly (A:U) tested: 30, 125, 250, and 1000 μg/ml. No precipitation was seen with several concentrations of BSA or MBSA, nor with poly (A) or poly (U) alone.

A commercial preparation of fluorescein isothiocyanate conjugated (FITC) goat anti-rabbit immunoglobulin (IgG, IgA, IgM), from Behring Diagnostics (Lot F458AE. Molar F/P ratio 3.0), was used. The FITC goat antiserum was absorbed twice using Balb/Aj thymocyte and spleen cells, diluted 1:3 with 0.01 *M* PBS, and stored at −20°. DEAE-dextran was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J.

Cell suspensions. Following cervical dislocation, thymuses were excised from 4- to 6-week-old Balb/Aj mice and placed in modified salt solution (MSS). The tissues were teased, the suspensions were allowed to sediment, and the supernatant was removed and centrifuged at 1000 rpm for 10 min at 4°. The cells were washed twice with MSS and resuspended in Veronal buffered solution (VBS), pH 7.2, containing 10% heat-inactivated fetal calf serum (FCS). The cells were suspended at a count of 1.8–2.1 × 10^7 cells/ml.

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ISSN 0037-9727

quots of 0.2 ml of thymocyte cell sus-
on were placed into small glass tubes.
enth of a milliliter of poly (A:U), 500
l, was added to the tube, and the sus-
on was incubated at 4° for 10 min. The
nsion was then centrifuged for 6 min at
rpm at 4°, and the supernatant was
ded. The suspension was washed twice
excess of VBS plus 10% FCS. After
ells were pelleted, 0.10 ml of a 1:16
on of antiserum to poly (A:U) was
l to the tube. The mixture was incu-
for 10 min at 4°, centrifuged as de-
d, and then washed twice in excess
plus 10% FCS. Following pelleting,
ml of FITC goat anti-rabbit immuno-
lin was added to the tubes and incu-
for 10 min. The mixture was centri-
and washed twice with excess VBS
10% FCS.

nunofluorescence. A Carl Zeiss fluo-
nce microscope was used. The cell sus-
ons were examined under ultraviolet
at a magnification of 400×, and at least
ells were counted per sample.

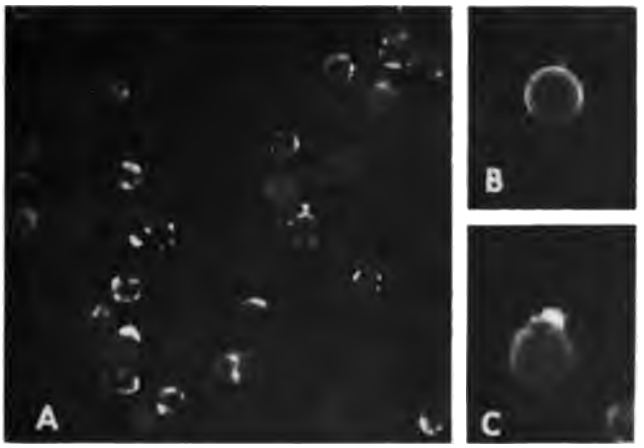
ntrols. Each experiment included a con-
ell suspension to which poly (A:U) was
dded. In addition, control samples with
vithout poly (A:U) were examined in
the serum of a normal nonimmunized
t, diluted 1:16 and absorbed as de-
d, was substituted for the antiserum to
(A:U).

ults. Uptake of poly (A:U) by thymo-
cytes. A mean of 80 ± 4% thymocytes ex-
hibited membrane-bound poly (A:U) in six
experiments. The patterns of staining ob-
served included ring formation, speckling,
and capping (Fig. 1). Those cells displaying
a homogeneous stain were not counted and
were considered nonviable. The percentage
of fluorescent cells in any control sample
never exceeded 3%. Cell viability consist-
ently exceeded 95% as determined by try-
pan blue dye exclusion.

The data of Table I show that the uptake
of poly (A:U) by thymocytes increased as
the concentration of (A:U) increased. Only
6% of cells stained at a concentration of 5
μg/ml of poly (A:U), whereas 44% of the
thymocytes stained at a concentration of 50
μg/ml. A maximum of 84% of cells stained
at 500 μg/ml; this percentage was not raised
by a 10-fold increase in concentration.

When thymocytes were preincubated with
either poly (A) alone, 500 μg/ml, or poly
(U) alone, 500 μg/ml, and then washed,
and incubated with poly (A:U), there was
no decrease in the number of stained cells
nor any alteration in the pattern of the stain.

In order to ascertain what effect the dura-
tion and temperature of incubation would
have on the degree of fluorescence uptake
by thymocytes, the cell suspensions were
incubated for 1, 10, 30, and 60 min at either
4 or 37°. The remaining steps of the proce-
dure were conducted at 4°. As indicated in
Table II, the temperature at which incuba-



. 1. Microphotograph of fluorescent thymocytes. (A) Field of thymocytes previously incubated with poly
and rabbit antisera to poly (A:U) then counterstained with goat FITC anti-rabbit Ig. Observe the speckled
1. (B) Ring pattern. (C) Capping. (400×).

tion was carried out as well as the duration of incubation had little effect on the membrane uptake of poly (A:U) by the thymocytes. A repeat of this experiment yielded essentially the same data.

Uptake of poly (A:U) by cortisone-resistant thymocytes. The uptake of poly (A:U) by the thymocytes of steroid-treated mice was examined. One-month-old Balb/Aj mice were injected intraperitoneally with 2.5 mg of hydrocortisone succinate on 2 consecutive days. The thymuses were removed on the third day, and cell suspensions were obtained in the usual manner. The number of cells demonstrating fluorescent staining was reduced markedly in hydrocortisone-treated mice in contrast to the thymocytes taken from noninjected control littermate mice (Fig. 2).

Inasmuch as steroids are known to stabilize the membranes of cells, a further experiment was conducted to determine if steroid uptake or coating of the membrane may have been responsible for the reduction in

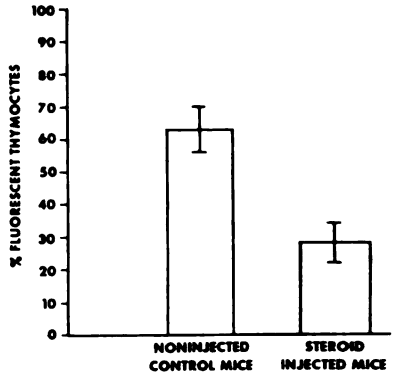


FIG. 2. Alteration in the number of fluorescent thymocytes in mice injected with hydrocortisone succinate. The two bars represent the mean of four paired experiments. The bar on the left indicates that 63 ± 7% of the thymocytes taken from uninjected control littermate mice showed fluorescence. The bar on the right reveals that 28 ± 6% of the thymocytes from steroid-injected mice showed fluorescence. This difference was significant using Student's paired *t* test ($P < 0.005$).

TABLE I. CORRELATION OF POLY (A:U) CONCENTRATION AND NUMBER OF FLUORESCENT THYMOCYTES.

Concentration of poly (A:U)[a] (μg/ml)	Fluorescent thymocytes (%)
0	3
5	6
50	44
500	84
5000	76

[a] Concentration of poly (A:U) in 0.1 ml added to 0.2 ml of a 1.8–2.1 × 10^7 cells/ml of thymocyte cell suspension.

TABLE II. EFFECT OF TEMPERATURE AND DURATION OF INCUBATION ON NUMBER OF FLUORESCENT THYMOCYTES.[a]

Incubation time (min)	Temperature (°C)	Fluorescent thymocytes (%)
1	4	67
1	37	78
10	4	81
10	37	72
30	4	74
30	37	78
60	4	78
60	37	71

[a] In each experiment, 0.1 ml of poly (A:U), 500 μg/ml, was added to a 0.2-ml aliquot of thymocyte cell suspension 1.8–2.1 × 10^7 cells/ml.

fluorescein uptake rather than the removal of steroid-sensitive thymocytes. Accordingly, thymocytes from normal control mice were incubated in VBS plus 10% FCS or, in this medium plus 100 μg/ml of hydrocortisone succinate for 20 min prior to incubation with poly (A:U). There was no difference in the uptakes of fluorescein stain between nonsteroid-treated cells (68%) and steroid-treated cells (60%). In addition, the degree of fluorescein uptake was not influenced by a 10-fold higher concentration of hydrocortisone.

Influence of DEAE-dextran on uptake of poly (A:U). Penetration of FLS Swiss mouse ascites tumor cells and human lymphocytes by poly (A:U) has been shown to increase 20-fold when the cells are pretreated with DEAE-dextran (3). In an attempt to ascertain whether DEAE-dextran pretreatment would increase the number of fluorescent cells or alter the pattern of staining of mouse thymocytes, the following experiments were conducted. In the first, thymocytes were pretreated for 15 min with DEAE-dextran at either 20 or 200 μg/ml, and then washed several times before poly (A:U) was added. Thymocytes from the same cell suspension, not pretreated with DEAE-dextran, were used as controls.

was no significant difference in the
:r of positive-staining cells between
o sets of cells (no pretreatment, 74%;
atment with 20 μg/ml of DEAE-dex-
30%; pretreatment with 200 μg/ml of
E-dextran, 70%). The morphologic
rance of the fluorescent cells was iden-
o those fluorescent thymocytes not
ated with DEAE-dextran.
illustrated in Table III, addition of
E-dextran and poly (A:U), simultane-
reduced the number of positive cells.
ıld appear that the positively charged
E-dextran may have reacted with the
vely charged poly (A:U), precluding
e by the thymocytes.
ping. As mentioned earlier, the poly
), anti-poly (A:U) complex was found
) when incubation was carried out at
n order to quantitate the frequency of
ıg of the thymocytes, these cells were
ıted at 4 or 37° in RPMI-1640 for
ls of 2 and 2.5 hr. Examination of the
ncubated at 4° for either time period
ed the same percentage of stained
ınd pattern of staining. Minimal cap-
ccurred at 4°. On the other hand, cells
ıted at 37° uniformly exhibited cap-
In addition, after incubation, the num-
f stained cells decreased from 72 to 57% at 2 hr, and to 39% at 2.5 hr (Table IV).

Discussion. Cells responding to the adjuvant action of poly (A:U) include the macrophage, T and B lymphocytes, stem cells, and memory cells (1). In addition, nonimmunocompetent cells such as those in the parotid gland and pancreas have demonstrated enhanced function under the influence of those polynucleotides (1). Inasmuch as the immune response to low numbers of thymocytes was enhanced remarkably by poly (A:U) (4), the binding of the adjuvant to these cells was measured in this initial study. The data show that the majority of mouse thymocytes possess membrane receptor sites for poly (A:U), and that a subpopulation of cells, presumably the more mature medullary thymocytes, bind poly (A:U) to a much lesser degree than the cortical thymocytes. DEAE-dextran appeared not to enhance or induce membrane staining of those thymocytes which by themselves did not bind poly (A:U).

In previous studies in our laboratory, the steroid-resistant (medullary) thymocyte has been implicated as one of the cells involved in the expression of the adjuvant action of polynucleotide complexes (5). In addition, steroid-resistant thymocytes have been shown by others to be fully capable of immunological response despite marked diminution in numbers of steroid-sensitive (cortical) cells (6). However, in this investigation, only 20–30% of thymocytes from steroid-treated mice revealed the presence of poly (A:U) on the membrane surface. This apparent paradox might be explained with the assumption that poly (A:U) may pene-

ABLE III. EFFECT OF COMBINED DEAE-
TRAN AND POLY (A:U) INCUBATION ON THE
NUMBER OF FLUORESCENT THYMOCYTES.

DEAE-dextran[a]		Poly (A:U)	Fluorescent thymocytes (%)
200 μg/ml	20 μg/ml	500 μg/ml	
+	–	+	10
–	+	+	30
–	–	+[b]	79
–	–	–[c]	3
+	–	+	11
–	+	+	25
–	–	+	69
–	–	–	2

ıe concentrations of DEAE-dextran used when
was added together with 0.1 ml of poly (A:U),
ml of thymocyte cell suspension, 3.6–4.0 × 10^7
l for a total volume of 0.3 ml.
ere, 0.1 ml of VBS was added in place of
-dextran to 0.1 ml of poly A:U and 0.1 ml of
yte cell suspension, 3.6–4.0 × 10^7 cells/ml.
total of 0.2 ml of VBS was added to 0.1 ml of
yte cell suspension.

TABLE IV. INFLUENCE OF TIME AND TEMPERATURE ON CAPPING OF POLY (A:U) ON THYMOCYTES

Duration of incubation after fluorescein staining (min)	Temperature (°C)	Percentage of fluorescent thymocytes		
		Speckled, ring	Cap	Total
0	4	77	4	81
120	4	72	6	78
150	4	74	5	79
0	37	69	3	72
120	37	4	53	57
150	37	3	36	39

trate the medullary thymocyte membrane via endocytosis without an intermediary stage of attachment to the membrane itself. Schell (7) showed that poly (A:U) entered the Ehrlich ascites tumor cell without separation of base pairing or breaking of the strands. Confirmatory work by Fenster *et al.* (3) indicated that poly (A:U) was rapidly taken into human lymphocytes and mouse ascites tumor cells and could be found as intact double-strand complexes in the nucleus and cytoplasm. In contrast, poly (A:U) may attach to membrane receptor sites available on cortical thymocytes with ingestion occurring over a longer period of time. If this hypothesis is correct, then, adjuvant activity ought to be associated with penetration of the plasma membrane by the complex.

The immunofluorescent technique used in these experiments detected the presence of poly (A:U) on the membrane only, and not intracellularly. Previous attempts to study the fate of poly (A:U) on cells by Schell (7, 8) and Fenster *et al.* (3) utilized isotopic techniques which enabled them to trace the fate of poly (A:U) intracellularly.

Fenster *et al.* (3) demonstrated that DEAE-dextran-pretreated ascites tumor cells and human lymphocytes increased penetration by poly (A:U) by 20-fold. In the present study, DEAE-dextran caused no increase in the number of thymocytes with membrane-detectable poly (A:U). Thus, these contrasting results suggest that membrane penetration of the medullary thymocyte may be enhanced by DEAE-dextran without requiring initial receptor site attachment to the membrane. Accordingly, medullary thymocyte membranes, by virtue of the maturation process, may demonstrate membrane dynamics that differ from the cortical thymocytes, i.e., the presumed blastic changes exhibited by medullary thymocytes and subsequent membrane alterations.

The present investigation would suggest that there may be at least two mechanisms of cellular uptake by mouse thymocytes of poly (A:U). One would involve a rapid penetration through the membrane by t ble-stranded complex, as in the s Fenster *et al.* (3), and another, by tion to the receptor sites on the mem of different cell types with subsequer brane activation, capping, and inge the cell. These receptors appeared for double-stranded polynucleotides they could not be blocked by single s poly (A) or poly (U).

Preliminary studies indicate that l peritoneal exudate cells, rat liver ce human peripheral T lymphocytes have surface receptors for poly (A:

Summary. Indirect immunofluor revealed that 80 ± 4% of isolated thymocytes were found to have me uptake of poly (A:U). Mice previc jected with hydrocortisone demons reduction to 20–30% in numbers of cent thymocytes. Preincubation of cytes with DEAE-dextran did not the percentage of fluorescent cells.

These findings suggest that medull mocytes bind poly (A:U) to a lesse than cortical thymocytes. Several nisms of thymocyte uptake of poly are suggested including adsorption membrane and/or rapid pen through the membrane.

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Received May 28, 1976. P.S.E.B.M. 1977,

GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 269-273 (1977)

ase of Marker Lysosomal Enzymes by 3-Methylindole and Indole from Rabbit Lung Lavage Cells (39652)[1]

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oduction. 3-Methylindole (3MI) is a olite produced during *in vitro* incuba- f tryptophan, indoleacetic acid, and indolic compounds with ruminal fluid d, in cattle, after intraruminal doses of tophan (2). 3-Methylindole has been to cause acute pulmonary edema and itial emphysema in cattle and goats . Clinical signs of pulmonary disease r within one to several hours after istration, and these animals often die a few hours to days depending upon se and method of administration (3, ulmonary edema is a predominant change in the development of pulmo- esions.

ole and 3MI can rupture biological ranes, and these effects may be re- to the lipophilic properties of these s. Indole and 3MI cause cell immobil- d disintegration of the membranous ure of ciliated ruminal protozoa (5). and other aryl compounds hemolyze ocytes (6) and cause lesions in rabbit joints which suggest membrane dam- , 8). High concentrations of 3MI in- ate between fatty acid chains of leci- icelles near the polar head which al- icelle structure (9) and induces struc- perturbations in lipid and protein re- of erythrocyte membranes (10, 11). teases from leukocytes, alveolar mac- ges, and other sources have been to cause pulmonary lesions (12–16). lysosomes are intracellular and the es are contained by lysosomal mem- s, this study was conducted to deter- (a) whether 3MI and indole would cause the release of marker lysosomal enzymes from rabbit lung lavage cells (LLC) *in vitro*, (b) and whether specific chemical agents would modify the effects of 3MI on LLC.

Materials and methods. Experiment 1. New Zealand white rabbits of both sexes, weighing about 2.5 kg, were used to harvest LLC by the method of Myrvik *et al.* (17) as modified by Tsan and Berlin (18). These techniques have been shown to yield at least 90% alveolar macrophages and small numbers of polymorphonuclear cells and lymphocytes (17, 18). Cold modified Hanks' solution (MHS) was used for washing out free lung cells and, for cell suspensions (approximately 70×10^6 cells were obtained from each rabbit). Cells were counted with a hemocytometer, and a suspension of 20×10^6 cells per ml was prepared.

One milliliter of a cell mixture containing 4×10^6 cells and 0, 200, 300, and 400 ppm (0, 1.53, 2.29, and 3.05 m*M*) of 3MI[3] or 0, 400, 600, and 800 ppm (0, 3.42, 5.13, and 6.84 m*M*) of indole[3] were incubated for 0, 1, 2.5, and 4 hr at 37° in a shaking water bath. The cell suspensions were then centrifuged for 10 min at 1200*g*. Two marker lysosomal enzymes, β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) and β-galactosidase (β-D-galactoside glucuronohydrolase, EC 3.2.1.23) were assayed in the supernatant by the method of Yarborough *et al.* (19). Soluble enzyme activity after incubation was expressed as a percentage of total enzyme activity released by sonication of unincubated cell suspensions.

Experiment 2. The effect of chemical agents[4] on the 3MI-induced release of β-galactosidase from LLC was tested using acetylcholine, acetylsalicylic acid, brain ex-

ntific Paper No. 4649. College of Agriculture h Center, Washington State University, Pull- ashington. Project 1893. This work was sup- in part by NIH Grant No. HL-13645.

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ISSN 0037-977

tract, cortisone, dimethylsulfoxide (DMSO), L-epinephrine, lysolecithin, phosphatidyl serine, retinoic acid, sphingomyelin, D-α-tocopherol, and Triton X-100. Selected amounts of acetylsalicylic acid, cortisone, retinoic acid, and D-α-tocopherol were dissolved in DMSO and then mixed with MHS so that a final concentration of 5% DMSO was used in all incubation mixtures with these compounds. The remaining compounds were dissolved in MHS. Sonication was used to make emulsions of brain extract, lysolecithin, phosphatidyl serine, and sphingomyelin. The final incubation mixtures were made by mixing 0.2 ml of LLC suspension (4×10^6 cells), 0.8 ml of 500 ppm of 3MI [(363 ppm) (2.77 m*M*) final concentration], and 0.1 ml of each agent. Mixtures were incubated at 37° in a shaking bath for 2.5 hr. β-Galactosidase activity was measured, and the soluble enzyme activity in the supernatant was expressed as in Expt 1. The enzyme activity released from cells in a control tube, without 3MI and chemical agents, was subtracted to give the net enzyme release. The effect of DMSO on the activity of solubilized β-galactosidase was tested in sonicated cell suspensions with or without 5% DMSO. The data were analyzed using Duncan's new multiple range test and Student's *t*-test for significant differences due to treatment for Expts 1 and 2, respectively.

Results. Experiment 1. The total activities of β-galactosidase and β-glucuronidase released by sonication from LLC were 589 ±

TABLE 1. RELEASE OF β-GALACTOSIDASE (β-GAL) AN LAVAGE CELLS BY INC

Incubation time (hr)	Enzyme	P 0
0	β-Gal	2.0 ± 1.4[a]
	β-Glu	1.9 ± 1.9
1	β-Gal	3.3 ± 1.6
	β-Glu	3.1 ± 0.7
2.5	β-Gal	3.7 ± 1.6
	β-Glu	4.1 ± 2.7
4	β-Gal	5.5 ± 1.8
	β-Glu	3.6 ± 2.5

[a] Each value is the mean ± SEM of three experime different from each other ($P < .05$).

LE II. RELEASE OF β-GALACTOSIDASE (β-GAL) AND β-GLUCURONIDASE (β-GLU) ACTIVITIES FROM LUNG LAVAGE CELLS BY INCUBATION WITH INDOLE.

›ation ne ır)	Enzyme	Percentage of total enzyme activity: Indole concentration (ppm) 0	400	600	800
	β-Gal	2.8 ± 1.0[a]			
	β-Glu	2.2 ± 2.2			
	β-Gal	2.3 ± 1.2	6.3 ± 1.2	10.2 ± 0.6	24.8 ± 8.9
	β-Glu	2.5 ± 0.9	8.7 ± 2.9	12.1 ± 3.4	18.7 ± 5.2
.5	β-Gal	3.7 ± 1.2	7.8 ± 1.3	18.1 ± 2.0	34.3 ± 6.6
	β-Glu	4.6 ± 2.4	11.8 ± 4.7	18.4 ± 6.0	25.5 ± 6.9
	β-Gal	4.8 ± 1.9	10.7 ± 2.1	24.1 ± 2.9	38.8 ± 5.7
	β-Glu	4.2 ± 2.1	19.6 ± 5.2	26.3 ± 4.5	32.1 ± 6.2

:h value is the mean ± SEM of three experiments. Values not sharing the same line are significantly : from each other ($P < 0.05$).

than the control at 2.5 hr with 600 nd at all incubation times with 800 f indole. After a 4-hr incubation, 4.8, 24.1, and 38.8% of the activity was ized at 0, 400, 600, and 800 ppm of , respectively. The same trends were t for the release of β-glucuronidase y by indole.

eriment 2. The effect of freezing and g cell suspensions in the presence and e of indole was tested, and the β- sidase activity released was com- to sonication. Enzyme activity re- by Triton X-100 (1000 ppm) was rable to that released by sonication. ng and thawing the LLC (two times) d 44% of the total β-galactosidase d by sonication, and indole (400 ppm ır) released only 15%. Freezing and g in the presence of indole resulted in oluble activity, indicating that indole ed the effectiveness of freezing and g in releasing this lysosomal enzyme. had no effect on enzyme activity : by sonication.

effects of 12 agents on the release of ctosidase activity from LLC in the ce and absence of 3MI are shown in III and IV. The incubation condi- of 2.5 hr and 363 ppm of 3MI and ıtrations of chemical agents were se- to produce moderate enzyme release. concentrations of Triton X-100, ıatidyl serine, and lysolecithin caused ant enzyme activity release when in-

TABLE III. MODIFICATION OF 3MI EFFECTS ON LUNG LAVAGE CELLS BY CHEMICAL AGENTS.

Treatment and concentration of agent[a]	Enzyme release[b] (Percentage of total activity): Without 3MI	With 3MI
Control	0[a]	20.1 ± 0.6[f]
Acetylcholine (18.2 ppm)	3.6 ± 0.4[a]	17.4 ± 0.9[g]
Brain extract (400 ppm)	4.0 ± 0.9[a]	21.2 ± 2.4[h]
L-Epinephrine (18.3 ppm)	1.0 ± 2.4[a]	20.1 ± 0.9[f]
Lysolecithin (400 ppm)	102.8 ± 4.4[b]	77.5 ± 5.7[g]
Lysolecithin (100 ppm)	67.1 ± 6.8[c]	52.7 ± 0.2[h]
Phosphatidyl serine (400 ppm)	8.9 ± 1.1[d]	33.2 ± 3.7[i]
Sphingomyelin (400 ppm)	6.7 ± 2.0[ad]	15.0 ± 1.5[j]
Triton X-100 (100 ppm)	22.4 ± 3.2[e]	67.2 ± 2.9[k]

[a] Four million LLC were incubated for 2.5 hr at 37° with or without 363 ppm (2.77 m*M*) of 3MI and chemical agents. Small amounts of enzyme release in control incubations were subtracted from all values to give net activity released. Each value is the mean ± SEM of three experiments.

[b] Means in each column with different superscripts are significantly different ($P < 0.05$).

cubated alone (Table III). In the presence of 3MI, Triton X-100 and phosphatidyl serine significantly increased soluble enzyme activity above that released by 3MI alone. Lysolecithin, in combination with 3MI, resulted in significantly more enzyme activity release than 3MI alone, but significantly less

TABLE IV. MODIFICATION OF 3MI EFFECTS ON LUNG LAVAGE CELLS BY CHEMICAL AGENTS DISSOLVED IN DMSO.

Treatment and concentration of agent[a]	Enzyme release[b] (percentage of total activity)	
	Without 3MI	With 3MI
Control	0[a]	20.1 ± 0.6[c]
DMSO (5%)	−1.2 ± 0.4[a]	7.4 ± 0.3[d]
Acetylsalicylic acid (60 ppm) + DMSO	0.1 ± 0.5[a]	47.4 ± 2.3[e]
Retinoic acid (10 ppm) + DMSO	4.8 ± 1.2[b]	14.2 ± 1.9[f]
D-α-Tocopherol (1.1 ppm) + DMSO	0.2 ± 0.8[a]	6.2 ± 2.3[d]
Cortisone (360 ppm) + DMSO	1.9 ± 1.4[ab]	10.9 + 3.5[cdf]

[a] Four million LLC were incubated for 2.5 hr at 37° with or without 363 ppm (2.77 m*M*) of 3MI and chemical agents. Small amounts of enzyme release in control incubations were subtracted from all values to give net activity released. Each value is the mean ± SEM of three experiments.

[b] Means in each column with different superscripts are significantly different ($P < 0.05$).

than lysolecithin alone at the concentrations used. With the exception of sphingomyelin which significantly reduced the effect of 3MI, the remaining compounds tested did not alter the effects of 3MI at the concentrations used.

Compared to the control without 3MI, only retinoic acid caused a small but significant enzyme release (Table IV). Dimethylsulfoxide significantly reduced enzyme release in the presence of 3MI from 20.1 to 7.4%. In the presence of 3MI, acetylsalicylic acid significantly increased soluble enzyme activity to 47.4% which was greater than DMSO, 3MI control, or acetylsalicylic acid alone. Retinoic acid significantly increased the soluble enzyme activity compared to the DMSO-3MI control (14.2 vs 7.4%). Dimethylsulfoxide (5%) had no effect on the activity of β-galactosidase in the assay system.

Discussion. The results of this study demonstrate that 3MI and indole can cause the release of β-galactosidase and β-glucuronidase from LLC. Whole cells were used, indicating that these indoles affect both cellular and lysosomal membranes in causing the release of the marker enzymes. The amount of enzyme activity solubilized is associated with the concentration of indole compounds and time of incubation. The time course and amount of β-galactosidase and β-glucuronidase released from LLC were similar during incubation with 3MI and with indole. Compared to 3MI, approximately two times the concentration of indole was required to give equivalent enzyme release. This suggests that the lipophilic properties of these indoles are important since the aqueous solubility of indole is approximately two times that of 3MI and it is less lipophilic than 3MI (6).

The data suggest that 3MI and indole cause enzyme release by rupturing cellular and lysosomal membranes. Release of β-galactosidase by 400 ppm of 3MI and 800 ppm of indole for 4 hr approximated the release by freezing and thawing the cell suspensions two times. β-Galactosidase and β-glucuronidase activities were released in similar relative amounts and in a similar time course. Indole nearly doubled the release of β-galactosidase from LLC subjected to freezing and thawing. This suggests that indole may enhance rupture of the LLC during freezing and thawing and/or promote the release of this enzyme activity from lysed cellular or lysosomal fragments.

The data in Table IV indicate that acetylsalicylic acid, Triton X-100, and phosphatidyl serine enhance the release of the lysosomal enzymes from LLC by 3MI, and that 3MI decreases the release caused by lysolecithin. Even though varying the concentrations of each chemical agent may affect the degree of enzyme release, the results indicate that some compounds enhance the release of lysosomal enzyme activity in the presence of 3MI and suggest that LLC may be more susceptible to lysis under these conditions.

The protective effect of DMSO was unexpected since it had been previously used as a carrier for water-insoluble compounds, and it was reported not to affect lysosomal stability or modify the effects of drugs on lysosomes (20).

The manner by which 3MI decreased the membrane-labilizing effect of lysolecithin is not known at this time. 3MI has been shown to bind with lecithin micelles *in vitro* (9) and may have interacted directly with lysoleci-

in the solution and reduced its effect on

mmary. Rabbit lung lavage cells (LLC) incubated with 0, 200, 300, and 400 (0, 1.53, 2.29, and 3.05 m*M*) of 3-ylindole (skatole, 3MI) or 0, 400, 600, 300 ppm (0, 3.42, 5.13, and 6.84 m*M*) dole for up to 4 hr at 37°. The release of lactosidase and β-glucuronidase activi-vas compared to the total enzyme activ-olubilized by sonication. Significant in-es in soluble enzyme activity were ob-d with these concentrations of 3MI, the amount of enzyme activity solubi-depended upon the 3MI concentration ncubation time. After incubation with ppm of 3MI for 4 hr, 58% of the total lactosidase and 46% of the β-glucuron-activities was solubilized from LLC. activity was greater than that released reezing and thawing two times. At r concentrations, indole caused a simi-lease of these lysosomal enzyme activi-Significantly more β-galactosidase ac-was released when LLC were incu-l with 3MI in combination with low ntrations of acetylsalicylic acid, reti-acid, Triton X-100, and phosphatidyl e than by incubation with each agent . Incubation with DMSO decreased ne release by 3MI, and 3MI inhibited of the membrane-labilizing effects of cithin. The data indicate that 3MI and e can cause the release of marker lyso-l enzymes from LLC and that the ef-of 3MI can be enhanced or decreased e presence of some other lipotrophic s.

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Received July 30, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 274-277 (1977)

Prostaglandin E_2 Effect from the Luminal Side on Renal Tubular ^{22}Na Efflux: Tracer Microinjection Studies (39653)[1]

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Renal prostaglandins (PG) have been implicated as possible mediators of the antihypertensive actions of the kidneys, perhaps through blunting vasoconstrictor responses or by increasing the renal elimination of sodium and water. PGE_2, the principal renal PG, is synthesized primarily in the medulla and papilla. However, several of the proposed mechanisms of its effect would require a cortical site of action. Prostaglandins of renal origin have been identified in the urine (1), and a tubular route of medullocortical PG transfer has been proposed. Previous studies in our laboratories (2), using tubular microinjection techniques, showed that about one-third of the PGE_2 reaching the loop of Henle was reabsorbed and did not re-enter the tubule; another third was temporarily delayed, and the remainder traversed it without tubular interaction. PGE_2 efflux beyond early distal puncture sites was very low (<14%). These studies suggested inhibition of sodium and water reabsorption in the distal nephron as a result of the action of PGE_2 at the luminal side rather than at the peritubular side. Alternatively, the PGE_2 that exited in the loop could have altered the function of the ascending tubule or some peritubular structures such as the efferent or afferent arterioles.

The present experiments were designed to examine the effects of PGE_2 from the luminal side on net tubular ^{22}Na efflux beyond the proximal convoluted tubule during 2.5% saline diuresis in rats. Sodium recovery was significantly increased by PGE_2. It is suggested that luminal PGE_2 action may represent an important component of the direct tubular effects of endogenous renal prostaglandins.

[1] This project was supported by NIH research Grant No. HL-14242, awarded by the NHLI, and AM-17711 awarded by the Division of GMS, USPHS/DHEW.

Methods. Male Wistar rats weighing 296 ± 31 g were anesthetized ip with Inactin, 100 mg/kg of body weight. Blood pressure was recorded from the cannulated right carotid artery. The animals were infused iv throughout the experiment with 2.5% saline at a constant rate of 0.05 ml/min to permit rapid serial urine collections. After an equilibration period of 45 min or longer, late proximal surface convolutions were punctured in the left kidney with sharpened volumetric micropipets having an outer tip diameter of 5–8 μm and an average volume of 39 ± 13 nl. Tubules were identified by lissamine green administration and, transit time was measured as described previously (2, 3). Each selected nephron was microinjected at the same site with a control and a PGE_2-containing radioactive solution. The radioactive mixtures were prepared as follows. (i) PGE_2 droplet: Isotonic saline, 0.5 ml, containing 100 μCi of [^{3}H]inulin was mixed with 0.5 ml of saline containing 100 μCi of ^{22}Na, and the solution was colored by adding 50 mg of lissamine green. This radioactive stock solution was mixed with a solution of PGE_2 in isotonic saline (10 μg/ml). The final PGE_2 concentration of the droplet was 5 μg/ml, and the average dose in a single intratubular injection was 200 pg.

(ii) The control droplet was prepared as the PGE_2 droplet except that the radioactive stock was mixed 1:1 with isotonic saline only.

The droplets were divided into small aliquots (0.01 ml each) and stored frozen in sealed glass capillaries. Each aliquot was used only once to prevent contamination or degradation of PGE_2. The injection rate was adjusted to maintain tubular diameter in the vicinity of the micropipet and to prevent retrograde flow. Injection rates averaged 29 ± 9 and 29 ± 8 nl/min for the control and

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ISSN 0037-9727

injections, respectively. Urine samre collected separately from the cathl left and right ureters into vials con- 10 ml of scintillation counting liquid). Ten 30-sec, three 60-sec, and one : consecutive samples were collected ıe left microinjected kidney, and four : samples from the right kidney. Toection time was 10 min from both

rence standards were prepared by ing a volume of radioactive mixture as the subsequently microinjected) directly into counting vials. Radicals were at least 98% pure as shown supplier (N.E.N. Boston, Mass.). In ıples, ^3H and ^{22}Na activities were ed simultaneously in a liquid scintilpectrometer with external standardias previously described (2–4). The method of preparation and sample ıg and the formulas used to calculate ues presented here have been previlescribed (2, 5–7). Data are preas the mean ± one standard deviaD). Statistical significance was evalu- the Student's *t*-test for paired varia-

lts. Forty technically successful mictions were made in 10 rats. Half of erved as controls and the other half ed PGE_2 in a concentration of 5 pg/ ldition to [^3H]inulin and $^{22}NaCl$. The e summarized in Table I. Mean sysılood pressure and the rate of urine ɔm the two kidneys were similar durtrol and PGE_2 injections. Late-proxid early-distal tubular transit times lso unaltered, suggesting that renal n was similar during the two injections. Injection time as well as pipet volume and injection rate (see Methods) were similar. Inulin recovery was complete in both cases, indicating that inulin, once deposited in the lumen, remains in the tubule and can be quantitatively recovered in the urine. ^{22}Na recovery from the left microinjected kidney was slightly, but significantly, higher when the injected solution contained PGE_2. The increase was even greater in total ^{22}Na recovery, revealing that PGE_2 significantly reduced the net sodium efflux in nephron segments beyond the late proximal puncture site.

In Fig. 1, the time course of inulin and sodium recoveries during control and PGE_2 injections are illustrated. Inulin recovery usually had a high peak and was complete within 5 min after ending the injection. The recovery pattern was similar for the control and PGE_2 injections. Recovery peaks were lower for ^{22}Na than for inulin in both control and PGE_2 injections. The appearance of sodium was somewhat delayed beyond inulin (30–60 sec), and its recovery terminated sooner (30–40 sec) in most cases. However, a temporal dissociation between ^{22}Na and [^3H]inulin could not be detected during either of the injections. The recovery lines did not cross on the upslope or downslope, nor could a difference be detected in mean tubular transit times of sodium and inulin. Precession of sodium over inulin was not expected or observed since these were luminal rather than close arterial injections.

In Fig. 2, the cumulative inulin and sodium recovery patterns are illustrated. Inulin recovery followed a similar course during control and PGE_2 injections and reached 100%. Sodium recovery, on the other hand,

TABLE I. EFFECT OF LUMINAL PGE_2 ON RENAL FUNCTION.

Function	Control	PGE_2	Δ	*P*
lood pressure (mmHg)	115 ± 17[a]	114 ± 17	1 ± 1	NS[a]
rine flow, left kidney (μl/min)	36 ± 8	37 ± 10	1 ± 1	NS
rine flow, right kidney (μl/min)	37 ± 12	38 ± 11	1 ± 1	NS
ate-proximal transit time (sec)	11 ± 1	11 ± 1	0	NS
arly-distal transit time (sec)	35 ± 5	35 ± 4	0	NS
ıjection time (sec)	83 ± 29	81 ± 27	2 ± 4	NS
ıulin recovery (%)	99.5 ± 3.2	100.8 ± 4.1	1.3 ± 1.1	NS
Na Recovery, left (%)	26.5 ± 12.4	28.9 ± 12.4	2.4 ± 1.4	<0.025
Na Recovery, total (%)	29.6 ± 13.3	34.1 ± 13.2	4.5 ± 1.7	<0.01

a are presented as mean ± SD. $N = 20$.
$P > 0.1$.

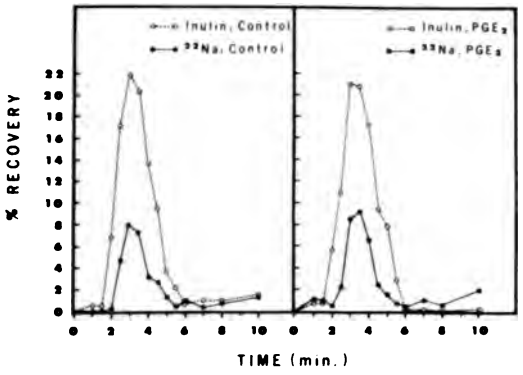

FIG. 1. Patterns of [³H]inulin and ²²Na recoveries after microinjection into late-proximal convolutions of the rat nephron with and without PGE_2. Diuresis was induced by iv infusion of 2.5% saline.

plateaued around 32% during the control injection and was increased to about 37% recovery when PGE_2 was administered simultaneously. This general pattern of reduced ²²Na efflux in the presence of PGE_2 was observed in 16 of the 20 paired injections. In some cases, the difference was marked, whereas in four injection pairs, a slight reverse effect was found, that is, PGE_2 appeared to increase ²²Na efflux slightly.

Discussion. The results of the present study reveal that PGE_2, applied to the luminal side of renal tubules, increases slightly, but significantly, the urinary recovery of simultaneously administered ²²Na. Since renal function and the method and site of injection were similar under the two conditions, the increased ²²Na recovery could best be attributed to its reduced net transtubular efflux under PGE_2 action. The inhibition of sodium efflux was low, about 3–4% of the amount injected. However, these rats were in intense hypertonic saline diuresis with markedly inhibited baseline sodium reabsorption as indicated by the high urine flow and ²²Na recovery of the control. It is difficult to reduce sodium transport further under these conditions. Nevertheless, the net sodium efflux decreased from 70 to 65%, or about 7% of the control value. In anti-diuresis, the same nephron segments may reabsorb more than 97% of the solute and water delivered to them and excrete less than 3%. Therefore, if the PGE_2 effect is of similar magnitude in anti-diuresis, luminal *action may account* for an increase in sodium excretion from less than 3% c delivery to more than 8%. This is sii the usual two- to three-fold increa served when PG synthesis goes from high, or when exogenous PGE_2 is into the renal artery.

Equal partition of medullary PG b peritubular blood and tubular fluid h reported (8), and PG of renal orig been identified in urine (1), indica cessibility of the renal tubule to PG. lar or other indirect effects of this PG should be minimal. It is therefore lated that luminal PGE_2 may modul tracellular fluid volume in the fo manner. A portion of the PGE_2 liber the medulla in response to a variety c uli enters the loop of Henle and is de via the tubular route to its site of where it reduces the net efflux of solu water. This luminal PGE_2 action may sent a significant component of the tubular effects of intrarenal prostagl

The mechanism of PG action on ²² flux is not apparent. It is likely th fraction of luminal PGE_2 previously to be temporarily delayed in the lo interacts with tubular cells and redu activity of a sodium pump or increa backleak of reabsorbed sodium. It sho emphasized that the effect of PGE_2 o recovery in the present studies was

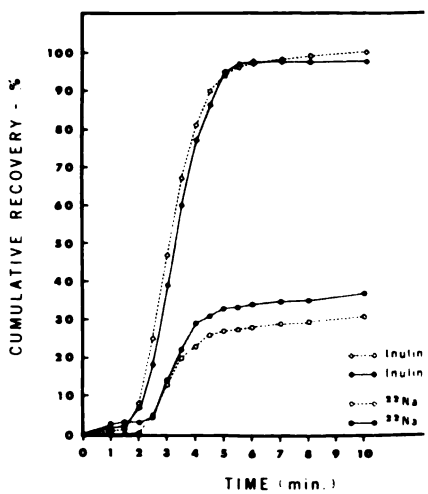

FIG. 2. Cumulative [³H]inulin and ²²Na r patterns during control injections and when PC incorporated into the microinjectate. Data are f same experiment as in Fig. 1.

ternate mechanisms of action of renal PG extracellular fluid balance should be con-ered. Redistribution of RBF, antagonism the renin–angiotensin aldosterone sys-n, altered efferent and afferent arteriolar ie, and, perhaps, a peritubular cortical : of action of distally absorbed PG may ntribute to the overall renal effects.

Summary. Using renal micropuncture hniques, the effect of luminal PGE_2 on : net efflux of simultaneously adminis-ed ^{22}Na was examined in rats in hyper-nic saline diuresis. [^3H]Inulin and $^{22}NaCl$:re microinjected into the lumen of late-oximal convoluted tubules with and with-it PGE_2, and the urinary recovery of ^3H id ^{22}Na were followed. Sodium recovery as significantly higher in the presence of PGE_2 while renal function and the method id site of injection were similar.

I wish to thank Dr. E. E. Elko and Dr. F. D. Sticht for the critical reading of this manuscript.

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Received July 19, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 278-284 (1977)

Hapten-specific Response to a New Arsonic Acid Conjugate, Arsanil Isothiocyanate[1] (39654)

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The discovery of the azobenzenearsonate (ABA) hapten system has greatly facilitated the study of delayed hypersensitivity by providing an immunodominant haptenic group toward which this type of immunity can be studied in a similar way to what has been done in the study of humoral immunity. In general, specificity for this immunodominant group has been studied by skin testing (1) and *in vitro* reactions correlating with delayed hypersensitivity, i.e., blast transformation of lymphocytes from sensitized animals on exposure to antigen and inhibition of macrophage migration in the presence of these lymphocytes and antigen (2, 3).

It was initially found that delayed hypersensitivity was directed mainly to the ABA group with an indication that there was a minor contribution by the aromatic ring of the amino acid carrier (4). Further experiments indicated that structural changes in the α-amino acid portion of the tyrosine residue produced considerable changes in immunogenicity and that the presence of the *N*-acetyl and carboxyl groups in ABA-*N*-acetyltyrosine (ABA-tyr) played a major role in the immunogenicity of the conjugates as well as in their ability to induce *in vitro* blast transformation of specifically sensitized lymph node cells (5). Other workers have demonstrated, with the use of defined tetrapeptides containing *p*-azobenzenearsonate-L-tyrosine, that immunocompetent cells are able to differentiate between these compounds, usually responding best to the tetrapeptide employed to initiate the immune response (6).

In this study, a new conjugate of arsanilic acid, the arsanil isothiocyanate (Ars-NCS) determinant, was tested. This determinant couples via an isothiocyanate group to form a thiourea link to the ϵ-amino group of lysine. The capability of this new material to sensitize guinea pigs and to elicit delayed hypersensitivity was studied as well as its ability to produce cross-reactions in animals sensitized to the ABA groups.

Materials and methods. Azobenzenearsonate (ABA). ABA conjugates were made by coupling the appropriate amounts of the diazonium fluoborate salt of arsanilic acid to the carrier as described previously (7).

Arsanilic isothiocyanate (Ars-NCS). Ars-NCS conjugates were made from *p*-arsanilic acid isothiocyanate synthesized and kindly provided by Dr. A. Soloway (Dey, A., and Soloway, A., in preparation). Ars-NCS was added gradually to the carrier protein dissolved in 0.1 *M* phosphate buffer, pH 8.5, until a molar ratio of 100:1 was reached. The mixture was stirred at room temperature for 18–20 hr, maintaining the pH at about 8.5. In the case of conjugates of bovine serum albumin (Ars-NCS-BSA), purification was effected by precipitation with trichloroacetic acid and resolubilization with 0.01 *N* NaOH, followed by dialysis against a 0.05 *M* phosphate buffer at pH 7.0. Conjugates of egg albumin (Ars-NCS-EA) were dialyzed directly without precipitation. Since isothiocyanate reacts only with free amino groups, a ninhydrin assay was done on the conjugates to see how many NH_2 groups were left uncoupled. Then, assuming approximately 60 free NH_2 groups per mole of BSA, the number reacting with Ars-NCS was calculated by difference and found to be between 7–15 groups per molecule for the various preparations studied. Since no difference in skin reactivity was seen at this level of conjugation they were all used without further distinction.

Ars-NCS conjugate of ϵ-amino caproic acid (Ars-NCS-EACA). This was made by dissolving 262 mg (2×10^{-3} moles) of ϵ-amino caproic acid in 20 ml of 0.2 *M* borate buffer (pH 9) and adding, with stirring, 259

[1] Supported by Grant AI-10895 from the U.S.P.H.S.

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ISSN 0037-9727

< 10^{-3} mole) of Ars-NCS. The pH usted to 9 with NaOH, and stirring tinued over night at room tempera-

olution was acidified with HCl and in ice; the resulting precipitate was ed by filtration and recrystallized iling water. The resulting solid, sol- acetone, was used without further cation.

als. Randomly bred albino female pigs weighing 300–400 g were used.

nization. Two groups of guinea pigs ed. One was immunized with 2 × le of ABA-tyr per animal contained 1-ml emulsion made with Freund's e adjuvant (8.5 parts mineral oil, ts arlacel-A, and 5 mg of killed tu- bacilli/ml), and distributed among footpads. The other group received ar emulsion containing Ars-NCS- at a dose of 2 × 10^{-6} mole per pig.

tests. Three weeks after immuniza- e animals were shaved, depilated, n tested with 30 μg each of ABA- BA-EA, Ars-NCS-BSA, Ars-NCS- d old tuberculin (OT, 1:400) in sa- imals used for *in vitro* experiments in tested with 1 μg of the ABA and S compounds prior to sacrifice to ne reactivity.

ites of skin tests were observed 3 hr r the presence of Arthus reaction, if d, subsequently, at 24 hr for meas- t of the average diameter and degree ration of the reaction.

ro stimulation of guinea pig lympho-

The guinea pigs were anesthetized her and bled to death by cardiac e. The popliteal, inguinal, axillary, scapular lymph nodes were removed sterile procedure. The pericapsular dissected free, and the lymph nodes ased with forceps in petri dishes con- culture medium kept cold. The cell ion was passed through a sterile wire and the cells were washed and resus- in culture medium. The culture me- ed was RPMI 1640 containing peni- 00 units/ml) and streptomycin (50 Associated Biomedic Systems, Inc., , N.Y.). Added were Kanamycin (2.5 mg/100 ml), 200 m*M* L-glutamine (0.5 ml/100 ml), and 10% heat-inactivated guinea pig serum (Grand Island Biological Co., Grand Island, N.Y.). Viable cell counts were made by the trypan blue dye exclusion method. Cell suspensions of 2 × 10^6 cells/ml were prepared, and 2-ml cultures were set up in triplicate in 12 × 75-mm sterilized glass tubes with loosely fitting aluminium caps. Antigens were added in 0.1-ml volumes to a final concentration of 12.5, 25, 50, 100, and 200 μg/ml. The cells were then cultured for a total of 72 hr in a 37° humidified incubator with an atmosphere of 5% CO_2 in air. Forty-eight hours after setting up the cultures, 1 μCi of [^{3}H]thymidine (Schwarz/Mann, Orangebury, N.Y., Specific activity: 3 Ci/mole) was added to each culture tube in a volume of 0.1 ml. The cultures were then harvested by washing once with phosphate-buffered saline, pH 7.4, at room temperature and twice with 10% trichloracetic acid (TCA) in the cold. A drop of 20% serum in saline was added as a carrier before adding TCA for the first time. The final precipitates were dissolved in 0.5 ml of Soluene-100 (Packard Instrument Co., Downers Grove, Ill.) per tube. These were transferred to glass vials containing 10 ml of scintillation fluid (16 g of PPO and 0.4 g of POPOP in 3.8 liter of toluene). The tubes were washed twice with 1 ml of the scintillation fluid which was added to the vials. The radioactivity was measured in a Packard liquid scintillation counter, and the counts per minute were recorded. The results were analyzed statistically by the Student's *t*-test, and differences which had a P value less than 0.05 were considered significant.

Results. In the first experiment, three guinea pigs were immunized with ABA-tyr and three with Ars-NCS-EACA in CFA. The skin tests were performed at 3 weeks with 30 μg of ABA-BSA and Ars-NCS-BSA and with OT 1:400. The results of the skin tests at 24 hr are presented in Table I.

It was seen that Ars-NCS-EACA was an immunogen capable of producing hapten-specific delayed hypersensitivity as all the animals gave strong delayed reactions averaging 18 mm of induration and erythema with Ars-NCS-BSA. There was a remarka-

ble degree of cross-reaction with ABA-BSA in these animals, the mean diameter of the reactions being 15 mm of induration and erythema.

The animals immunized with ABA-tyr showed, as previously, a positive reaction to ABA-BSA with a mean diameter of 16 mm of marked erythema and induration in all the animals as well as a central blanche in two of them. These animals also gave reactions averaging 13 mm of erythema and induration with the Ars-NCS-BSA. Thus, in both cases, cross-reaction was evident although the homologous conjugates elicited a somewhat more intense skin test. There were no Arthus reactions observed at 3 hr in any of these six animals. All reactions to OT were of comparable intensity.

The experiment was repeated with six more animals, and, this time, they were skin tested with 30 μg of egg albumin conjugates ABA-EA and Ars-NCS-EA. The results shown in Table II demonstrated essetially the same capability of Ars-NCS-EACA to function as an immunogen for hapten-specific delayed hypersensitivity. Three animals immunized with Ars-NCS-EACA responded to Ars-NCS-EA with a skin test having a mean diameter of 18 mm. The response to ABA-EA was approximately 8 mm with somewhat less induration. A cross-reaction was also observed in the case of animals immunized with ABA-tyr, the mean reaction observed with ABA-EA being 17 mm and with ABA NCS-EA, 15 mm. Again, in both instances, reactions to the homologous conjugates were somewhat larger and more intense.

The ability of lymphocytes from animals immunized with Ars-NCS-EACA to undergo *in vitro* stimulation by antigen was tested next. In the outbred population of guinea pigs used in this study, the response in terms of skin reaction with both type of conjugates were variable from animal to animal, but skin reactions were generally paralleled by *in vitro* responses to the same antigen. Therefore, animals which had a poor skin reaction to a test dose of 1 μg of the homologous conjugate and did not show significant stimulation *in vitro* are not presented in the study.

TABLE I. DELAYED REACTIONS TO ABA AND ARS-NCS CONJUGATES OF BSA IN GUINEA PIGS IMMUNIZED WITH ABA-TYR OR ARS-NCS-EACA IN COMPLETE FREUND'S ADJUVANT.

Guinea pig number	Immunized with	Delayed reactions to ABA-BSA (30 μg)		Ars-NCS-BSA (30 μg)		OT	(1:400)
1	ABA-tyr	14[a]	++[b]	7	±	12	++
2	ABA-tyr	17	+++	16	++	16	+++
3	ABA-tyr	17	+++	15	++	12	++
4	Ars-NCS-EACA	12	++	20	++	18	+++
5	Ars-NCS-EACA	20	++++	17	++	22	++++
6	Ars-NCS-EACA	14	++	17	++	12	++

[a] Diameter of erythema in millimeters.
[b] Degree of induration.

TABLE II. DELAYED REACTIONS TO ABA AND ARS-NCS CONJUGATES OF EGG ALBUMIN IN GUINEA PIGS IMMUNIZED WITH ABA-TYR OR ARS-NCS-EACA IN COMPLETE FREUND'S ADJUVANT.

Guinea pig number	Immunized with	Delayed reactions to ABA-Ea (30 μg)		Ars-NCS-Ea (30 μg)		OT	(1:400)
1	ABA-tyr	16[a]	++[b]	12	++	12	++
2	ABA-tyr	18	+++	17	+++	14	++
3	ABA-tyr	18	+++	15	++	14	+++
4	Ars-NCS-EACA	11	++	18	+++	16	+++
5	Ars-NCS-EACA	4	+	20	++++	18	+++
6	Ars-NCS-EACA	11	++	17	+++	13	+++

[a] Diameter of erythema in millimeters.
[b] Degree of induration.

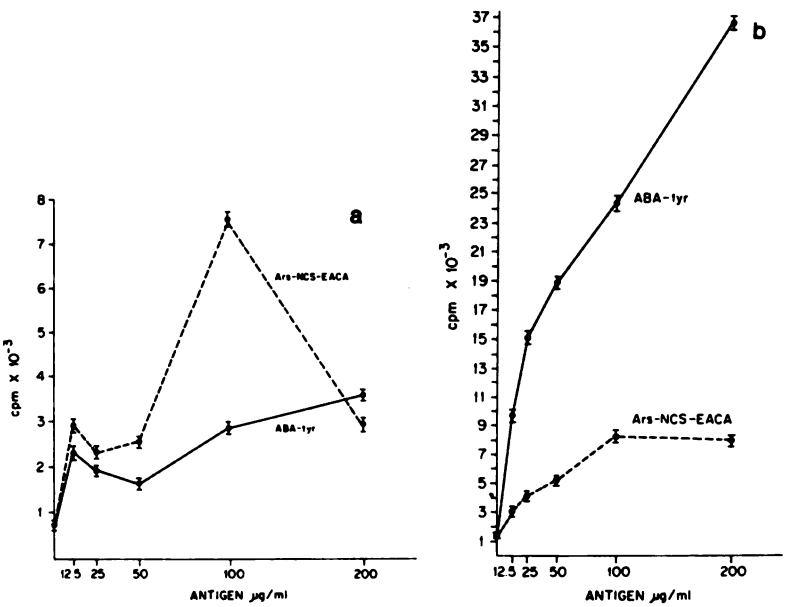
il dose-response curves to mono-
onjugates (Ars-NCS-EACA and
:) are seen for an animal immunized
-NCS-EACA (Fig. 1a) and an ani-
unized with ABA-tyr (Fig. 1b). In
tances, either monomeric antigen
l significant degrees of stimulation,
as apparent that the homologous
e used for immunization produced
mulation *in vitro*. When similar re-
to the polyvalent conjugates (Ars-
. and ABA-EA) were studied in a
pig immunized with Ars-NCS-
Fig. 2a) or with ABA-tyr (Fig. 2b),
ain apparent that a large degree of
ctivity was present, in that both
es produced good responses with
; of lymphocytes. This time, how-
s difference was seen between the
ous and heterologous conjugate.

effort to determine whether the
y or degree of cross-reaction be-
Ars-NCS and ABA conjugates
with time, groups of guinea pigs
ed with either Ars-NCS-EACA or
were sacrificed at intervals up to
, and their lymphocytes were cul-
tured *in vitro* with the various conjugates to measure [^{3}H]thymidine incorporation.

The results shown in Table III and IV are the means of triplicate samples at the particular dose which showed maximum stimulation for that particular antigen (usually 100 or 200 μg/ml). In virtually all cases, stimulation with the homologous monomeric conjugate was best and a comparison of homologous conjugate stimulation to heterologous conjugate stimulation gave ratios averaging 12:1 (range 2–42) for both sets of immunized pigs. Stimulation measured with the polyvalent conjugates did not show the same consistent trend toward favoring the homologous conjugate, and ratios of stimulation of homologous to heterologous polyvalent conjugates in both groups of pigs were closer to 1:1 (range 0.17–6.7).

There was no clear cut change in stimulability with time either in the degree of stimulation by the homologous conjugate or in the nature of the cross-reactivity between the two types of conjugate.

Discussion. Guinea pigs immunized with Ars-NCS-EACA in complete Freund's adjuvant became sensitized for a delayed hy-



Typical dose-response curve to Ars-NCS-EACA and ABA-tyrosine *in vitro* by cells from a guinea pig with Ars-NCS-EACA (a) and ABA-tyr (b). the results are expressed as the (mean of the counts/ 0^{-3}) ± the standard error of the replicate samples. The dose of antigen is expressed in micrograms/ culture medium.

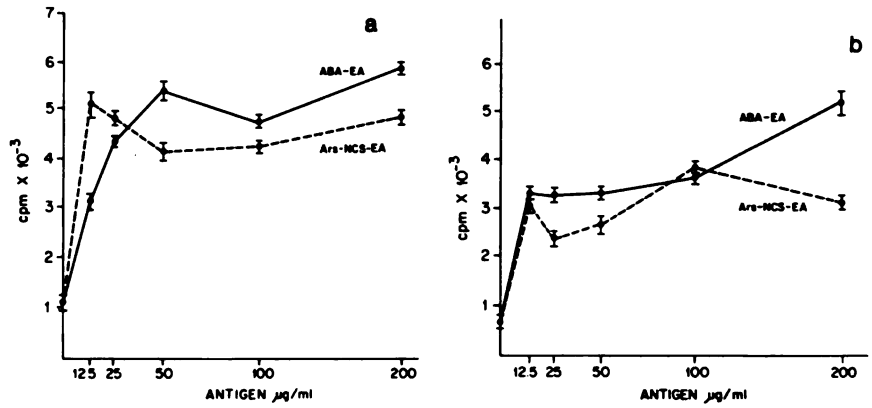


FIG. 2. Typical dose-response curve to ABA-EA and Ars-NCS-EA *in vitro* by cells from a guinea pig immunized with Ars-NCS-EACA (a) and ABA-tyr (2b). The results are expressed as the (mean of the counts/minute × 10^{-3}) ± the standard error of the replicate sample. The dose of antigen is expressed in micrograms/milliliter of culture medium.

TABLE III. ANTIGEN-INDUCED [^{3}H]THYMIDINE INCORPORATION BY LYMPHOCYTES FROM GUINEA PIGS IMMUNIZED WITH ARS-NCS-EACA AND STIMULATED WITH ABA AND ARS-NCS CONJUGATES.[a]

Guinea pig number	Time of testing (weeks)	Immunized with	Antigen used *in Vitro*	Radioactivity (mean cpm of test − mean cpm of control)	Ratio of homologous:heterologous conjugate stimulation	
					Monovalent	Multivalent
1	4	Ars-NCS-EACA	ABA-Tyr	268 (N.S.)[b]	38.2	
			Ars-NCS-EACA	10,229		
			ABA-BSA	11,833		0.19
			Ars-NCS-BSA	2,278		
2	5	Ars-NCS-EACA	ABA-Tyr	768	6.2	
			Ars-NCS-EACA	4,733		
			ABA-EA	4,758		0.85
			Ars-NCS-EA	4,060		
3	5	Ars-NCS-EACA	ABA-Tyr	2,233	8.0	
			Ars-NCS-EACA	17,932		
			ABA-EA	635		6.3
			Ars-NCS-EA	4,029		
4	7	Ars-NCS-EACA	ABA-Tyr	2,595	2.5	
			Ars-NCS-EACA	6,606		
			ABA-BSA	1,575		5.0
			Ars-NCS-BSA	7,955		
5	12	Ars-NCS-EACA	ABA-Tyr	2,937	7.7	
			Ars-NCS-EACA	22,582		
			ABA-BSA	441 (N.S.)		5.9
			Ars-NCS-BSA	2,645[c]		
					Average 12.5	3.6

[a] The result for each antigen is the mean of triplicate samples at the dose of antigen which gave the maximum incorporation.

[b] N.S., not significant.

[c] *P* value of <0.05. All other differences have a value <0.01.

persensitivity which could be elicited with other arsanil conjugates. These conjugates were of two types, those in which the arsanilic group was coupled via an NCS linkage to lysine and others, in which the diazo linkage to tyrosine and histidine was present. Marked cross-reactivity in the *in vivo* response was observed. For example, a group of animals immunized with Ars-NCS-EACA gave an average reaction of 18 mm

TABLE IV. ANTIGEN-INDUCED [^{3}H]THYMIDINE INCORPORATION BY LYMPHOCYTES FROM GUINEA PIGS IMMUNIZED WITH ABA-TYROSINE AND STIMULATED WITH ABA AND ARS-NCS CONJUGATES.[a]

Guinea pig number	Time of testing (weeks)	Immunized with	Antigen used *in Vitro*	Radioactivity (mean cpm of test − mean cpm of control)	Ratio of homologous:heterologous conjugate stimulation	
					Monovalent	Multivalent
1	4	ABA-Tyr	ABA-Tyr	35,396	5.1	
			Ars-NCS-EACA	6,877		
			ABA-BSA	18,787		
			Ars-NCS-BSA	−364 (N.S.)[b]		
2	5	ABA-Tyr	ABA-Tyr	2,289	8.8	
			Ars-NCS-EACA	258		
			ABA-EA	4,409		1.4
			Ars-NCS-EA	3,062		
3	6	ABA-Tyr	ABA-Tyr	40,898	3.0	
			Ars-NCS-EACA	13,393		
			ABA-BSA	8,337		1.1
			Ars-NCS-BSA	7,474		
4	9	ABA-Tyr	ABA-Tyr	15,681	42.0	
			Ars-NCS-EACA	372		
			ABA-EA	3,114		2.0
			Ars-NCS-EA	1,485		
5	12	ABA-Tyr	ABA-Tyr	47,253	2.0	
			Ars-NCS-EACA	23,569		
			ABA-BSA	677(N.S.)		.17
			Ars-NCS-BSA	3,903		
					Average 12.1	1.1

[a] The result for each antigen is the mean of triplicate samples at the dose of antigen which gave the maximum corporation.

[b] N.S., not significant; all other differences have a P value of >0.01.

liameter) with Ars-NCS-BSA and 15 mm liameter) with ABA-BSA. All these re-)onses were accompanied with marked er-:hema and induration. A similar cross-re-:tion was seen on skin testing animals hich had been immunized with ABA-tyr in)mplete Freund's adjuvant. A group of ıch animals responded with an average :in test diameter of 17 mm to ABA-NCS-A. Again, all these reactions were accom-anied by marked erythema and induration.

In vitro stimulation of lymph node lym-hocytes taken from animals immunized ith Ars-NCS-EACA and ABA-tyr was lso successful with antigens which included ıe monovalent compounds as well as the ıultivalent forms, ABA-BSA, ABA-EA, ırs-NCS-BSA, and Ars-NCS-EA, and, es-:ntially similar cross-reactivity was seen, ıus, further demonstrating the immuno-ominance of the arsonic acid group (4).

In vitro reactions tended to indicate a ner degree of discrimination than did *in ivo* reactions, especially when monovalent onjugates were used as the test antigens. Thus, lymphocytes from animals immunized with either ABA-tyr or Ars-NCS-EACA gave greater degrees of stimulation with the homologous conjugates. The average ratio of homologous to heterologous conjugate stimulation was about 12:1 in both instances (Table III and IV). However, these differences tended to disappear when multivalent conjugates were used, and the average ratios of homologous to heterologous conjugate stimulation were closer to unity.

The greater stimulation produced by the homologous monovalent conjugates could be a reflection of the contribution by the NCS or diazo linkage groups, as well as the lysine or tyrosine attachment residues to the immunogenic units. Similar extensions of the immunoreactive grouping beyond the ABA moiety were observed in other studies of cell-mediated immunity (3, 6, 8). Therefore, for example, if only the arsonic acid group of Ars-NCS-EACA was able to fit the T-cell receptor for the ABA-tyr immunogenic unit, insufficient binding affinity to trigger the cell might be generated. How-

ever, the possibility of multipoint attachment which could be achieved by the polyvalent protein conjugates may make triggering of cross-reactions more likely and, thus, may offer an explanation for smaller differences in reactivity of the multivalent compounds.

Reactivity, in terms of both skin tests and *in vitro* stimulation, was seen in animals up to 12 weeks (which was the longest duration studied). Some studies in rats and guinea pigs with ABA-tyr, ABA-histidine, and ABA-trypotophan (9, 10) have indicated that the fine specificity recognition beyond the ABA group (as tested by inhibition of macrophage migration in the presence of sensitized lymphocytes and antigen) is not seen as early as 3–4 weeks after immunization, but develops later. No such increase in sensitivity or 'immunological maturation' was observed in the present study: in fact, the fine specificity recognition was already present as early as 4 weeks. It is possible that examination of more animals over a longer period of time might provide some evidence of a "maturation" of the immune response, but the present data are insufficient to arrive at any conclusion other than the high degree of arsonic acid specificity seen with both types of conjugate.

Summary. A new derivative of arsonic acid, arsanil isothiocyanate was used to couple arsonic acid via an isothiocyanate group to amino groups. Immunization of guinea pigs with this determinant attached to ε-amino carproic acid in complete Freund's adjuvant led to the production of delayed hypersensitivity toward this haptenic determinant, as indicated by positive skin tests to compounds to which the determinant was coupled to egg albumin and bovine serum albumin. In addition, lymphocytes these immunized animals respond these antigens *in vitro* by undergoing transformation as measured by inco tion of tritiated thymidine.

The dominance of the arsonic acid in the determinant was seen in the fc positive skin tests as well as in *in vitro* lation with the various conjugates in a immunized with either Ars-NCS-EA ABA-tyr.

The response by the homologous pounds were, however, generally than those of the heterologous comp These results suggest an important co tion to the reactivity of the immunc nant arsonic acid group by the neigh site of attachment.

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Received September 1, 1976. P.S.E.B.M. 19 154.

DINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 285-288 (1977)

ızyme Immunoassay and Radioimmunoprecipitation Tests for the Detection of Antibodies to *Rochalimaea* (Rickettsia) *quintana* (39655)[1]

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troduction. Epidemics of trench fever, ed by *Rochalimaea* (Rickettsia) *quin-*, occurred during the First and Second ld Wars as well as the Spanish Civil , and the disease remains endemic in e areas of the world. The infective agent be isolated from the blood (1), and itive serological techniques are required the detection of specific antibodies. ıplement fixation tests (CF) with whole or soluble cytoplasmic antigens detect *uintana* antibodies (2), but lack of sensi- y has led to introduction of immunoflu- cent (IF) and passive hemagglutination A) techniques. However, in a seroepi- iological survey in an area suspected of :micity for trench fever, only 33% of people were positive by either IF or A tests, although the combined positives ɔoth were 55% of the total sampled ɔublished data). In an attempt to im- 'e sensitivity, a radioimmunoprecipita- test and an enzyme immunoassay test : developed and compared to IF and A for detecting *R. quintana* antibodies in m.

aterials and methods. Soluble antigen. s of *R. quintana*, strain Fuller (3) or dalupe (4), were grown on hemolyzed e blood agar for 5 days at 37° in a moist ɔsphere of 5% CO_2. The cells were har- ɜd in a sucrose-phosphate-glutamate tion (5), washed three times, resus- ɪed in 0.1 *M* phosphate-buffered saline ɜ), pH 7.2, and disrupted by sonication ɪ0 min at 1.5 A (Model 60 W Ultrasonic ntegrator, M.S.E., London). After cen- gation at 75,000*g* for 1 hr, the supernа- fluid served as the soluble antigen. The ɣen was stable at 4° for several months added thiomersol at a 1:10,000 final concentration. Protein concentration was estimated by use of the Folin-Ciocalteau reagent (6).

Sera. Animal sera. After collecting preimmunization sera, animals were inoculated with soluble or whole cell antigen. Equal 1-ml vol of soluble antigen from *R. quintana* (containing 300-500 μg of protein/ml) and Freund's complete adjuvant were mixed and inoculated sc into New Zealand white rabbits and Hartley strain guinea pigs. Rabbits were bled at 3 and 4 weeks. After 6 weeks, guinea pigs were given two further inoculations sc; serum samples were taken eight weeks after the first inoculation. For guinea pig antiserum to *R. quintana* (Fuller) whole cells, equal 1-ml vol of washed cells and Freund's complete adjuvant were mixed and inoculated sc again 6 weeks later. Serum samples were taken 8 weeks after the first inoculation.

Human sera. Sera were obtained from residents of the Nabeur region of Tunisia, an area suspected of being endemic for trench fever. Sera were also obtained from Boston area laboratory personnel.

Passive hemagglutination. Tanned sheep red blood cells (SRBC) were used in a standard method (7). SRBC were coated with *R. quintana* (Fuller) soluble antigen at 5 to 10 μg of protein per ml of SRBC. These were added to sera previously inactivated (56° for 30 min) and adsorbed with SRBC.

Immunofluorescence techniques. The indirect method was used. *R. quintana* whole cells were suspended in a solution of 5% (w/v) normal yolk sac in PBS (homogenized by use of a Waring blender) and fixed on glass slides with acetone. Test or control sera were added and incubated 30 min at 37°. Fluorescein isothiocyanate-conjugated antiglobulin (Difco Laboratores, Detroit, Mich.) was added and incubated for 30 min at 37°. The slides were washed with PBS,

ıupported in part by U.S. Army Contract DAMD- -C-5038.

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ISSN 0037-9727

mounted in glycerol, and observed under a Zeiss fluorescence microscope equipped with BG12 and BG38 exciter filters and an OG1 barrier filter.

Radioactive antigen. Soluble antigen of *R. quintana* (Fuller) was labeled with ^{125}I by use of the chloramine T method (8). $Na^{125}I$ (New England Nuclear, Boston, Mass.) was diluted with 0.5 *M* phosphate buffer, pH 7.5 to contain 10 mCi/ml. One millicurie (0.1 ml) was added to 0.4 ml of antigen (containing 1.2 mg/ml of protein) plus 1.1 ml of 0.05 *M* phosphate buffer, pH 7.4. Chloramine T (500 μg in 0.1 ml) was added, and the reactants were stirred at room temperature for 30 sec. Sodium metabisulphite (1 mg in 0.1 ml) plus 1 mg (0.1 ml) of KI was added to stop the reaction. Labeled material was partially purified by filtration on Sephadex G-50. Fractions were collected and assayed for radioactivity by counting ^{125}I disintegration in a Nuclear-Chicago automatic gamma counter. The labeled fractions were pooled and dialyzed for 48 hr against PBS to remove residual iodine. Precipitation with 5% trichloracetic acid showed that approximately 97% of the ^{125}I was protein associated. The sp act of the antigen preparations was 20 to 30 μCi/mg of protein.

Radioimmunoprecipitation test (RIP). The procedure was similar to those described by Gerloff *et al.* (9), Dalrymple *et al.* (10), and others. ^{125}I-Labeled antigen and sera were diluted in borate-buffered saline (BSB), pH 8.0; each serum was tested in triplicate. A 0.2-ml vol of test serum plus 0.1 ml of labeled antigen were incubated in plastic tubes at 37° for 1 hr. Anti-globulin (0.1 ml) was added, and the mixtures were incubated at 37° for 1 hr, followed by incubation at 4° for 18 hr. Controls consisted of normal serum plus labeled antigen plus anti-globulin; labeled antigen plus anti-globulin; and labeled antigen plus BSB plus anti-globulin. Test and control preparations were centrifuged at 4000*g* for 30 min. Samples (100 μl) of the supernatant fluids were assayed for radioactivity (cpm). The precipitates were washed three times with BSB by centrifugation and also counted. Binding of antigen, and hence, a positive serum, was calculated in two ways: (i) by the difference in the radioactivity (cpm) of washed precipitates of test and control samples, and (ii) by the percentage of radioactivity in the supernatant fluids, as described by Brunner and Chanock (11); $[(S_c - S_t)/S_c] \times 100 = X\%$, where S_t is the mean counts per minute in supernatant fluids from a test serum, and S_c the mean counts per minute in control supernatant fluids.

Enzyme-labeled anti-globulin. Globulin fractions of goat anti-human IgG and goat anti-rabbit IgG (Antibodies, Inc., Davis, Calif.) were obtained by $(NH_4)_2SO_4$ precipitation (40% saturation) of whole sera. The method for coupling the globulins with enzyme (horseradish peroxidase, type IV Worthington Biochemical Corp., Freehold N.J.) was by use of periodate (12). Peroxidase-labeled globulin was separated from unlabeled material by gel-filtration on 2.5 × 80-cm columns of Sephadex G-200. Fractions that gave maximum adsorption in a spectrophotometer at both 403 (enzyme) and 280 nm (protein) were pooled and precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation. The precipitate was suspended in a volume of PBS to give a protein concentration of approximately 2 mg/ml and dialyzed against PBS. The preparations were tested for immunologic reactivity in gel-diffusion plates against the appropriate globulin and stored at −4° until used.

Enzyme immunoassay. The procedure for solid-phase enzyme immunoassay (EIA) was based on the enzyme-linked immunosorbent assay of Engvall and Perlmann (13) as modified by Ruitenberg *et al.* (14). Soluble *R. quintana* antigen at 100 μg/ml in 0.1 *M* sodium carbonate buffer, pH 9.5, was added to round-bottom wells in polystyrene microtiter plates (Cooke Engineering, Alexandria, Va.), 0.2 ml/well. The antigen was absorbed 3 hr at 37°, plus 4 days at 4°. The antigen was removed, and 0.3 ml/well of carbonate buffer plus 1% (w/v) bovine serum albumin (BSA) was added.

The plates were incubated at 4° overnight and washed three times with distilled water plus 0.05% (v/v) Tween 20. Test and control sera, diluted in PBS, plus 0.05% (v/v) Tween 20 were added (0.05 ml/well) and incubated for 30 min at 37°. The plates were washed as above, and peroxidase-conju

anti-globulin was added (0.05 ml/
t a 1:100 to 1:200 dilution. The dilu-
the conjugates was PBS with 1% (w/
ı and 0.05% (v/v) Tween 20 added.
ates were incubated for 30 min at 37°
ıshed as above; 0.3 ml/well of sub-
[0.05% 5-amino salicylic acid in dis-
vater plus 0.005% H_2O_2, Ref. (14)]
ded. After 30–60 min at room tem-
re, the reaction was stopped with 0.1
1 *N* NaOH. A red-brown reaction
t was formed; the end points were
isually by comparison with controls
n plus normal serum and antigen plus

lts. Animal sera. A comparison of the
ity of hyperimmune animal sera in
A, RIP, and EIA is shown in Table I.
: third week after inoculation with
antigen, rabbit sera gave similar ti-
IF and PHA and slightly higher titers
and EIA. No differences were seen
n rabbit sera prepared against Fuller
dalupe strains of *R. quintana*. Higher
were demonstrated in guinea pigs
had received multiple inoculations.
ferences were noted between guinea
:isera prepared with whole cells and
vith soluble antigen, nor did use of
cells produce higher antibody titers
ɔluble antigen. Titers of preimmuni-
sera were <1:10 in all tests.
ıan sera. Results with 10 sera are
in Table II. Of these sera, six were
ɛ in IF tests, and seven were positive
in PHA tests. However, all 10 sera were positive in both RIP and EIA tests, with generally higher titers. Sera obtained from four other people in the same region of Tunisia were negative by all four tests (titers <1:10), as were two sera obtained locally from laboratory personnel. All sera were examined twice by RIP and EIA on different days: The variation was never more than one twofold dilution.

Discussion. Of 14 human sera (Tunisian) tested, totals of six and seven were positive in IF and PHA, respectively, whereas 10 sera reacted in both RIP and EIA. This indicates an increased sensitivity of these tests, even though individual titers of some positive sera may be the same or higher in PHA or IF. Although RIP and EIA each detected an equal number of positive sera, the advantages of EIA are ease of reagent preparation, simplicity and safety of use, and stability of label.

Most EIA techniques use plastic tubes rather than microtiter plates. The tubes allow for the easy reading of reactions spectrophotometrically, whereas plates require lower volumes of reagents. The latter is important if high concentrations of antigen are needed for optimal results and the supply is limited. Although some investigators have found that as little as 5 μg/ml of antigen will give a successful EIA test (14), and it is known that, for some proteins, concentrations above 10 μg/ml result in a decreased percentage of adsorption (15), we found

BLE I. REACTIVITY OF ANIMAL SERA IN IMMUNOFLUORESCENCE (IF), PASSIVE HEMAGGLUTINATION (PHA), RADIOIMMUNOPRECIPITATION (RIP), AND ENZYME IMMUNOASSAY (EIA) TESTS.

ıl	No.	Antigen	*R. quintana* strain[a]	Weeks post-inoculation	Antibody titer[b]			
					IF	PHA	RIP	EIA
	1	Soluble	G	3	640	320	1,280	2,560
				4	2,560	5,000	5,000	10,000
	2	Soluble	G	3	640	640	1,280	1,280
				4	640	640	640	1,280
	3	Soluble	F	4	640	160	1,280	2,560
	1	Whole cell	F	8	2,560	2,560	5,120	n.t.[c]
	2	Soluble	F	8	10,000	20,000	20,000	
	3	Soluble	F	8	10,000	20,000	20,000	
	4	Soluble	F	8	2,560	1,280	2,560	

ıdalupe (G), Fuller (F).
iprocal of serum dilution.
tested.

TABLE II. REACTIVITY OF HUMAN SERA TO *R. QUINTANA* ANTIGEN IN IMMUNOFLUORESCENT (IF), PASSIVE HEMAGGLUTINATION (PHA), RADIOIMMUNOPRECIPITATION (RIP), AND ENZYME IMMUNOASSAY (EIA) TESTS.

Serum No.	Antibody titer[a]			
	IF	PHA	RIP	EIA
703	<10	320	320	160
707	20	20	40	40
708	<10	80	80	80
714	<10	160	160	160
721	10	80	80	40
739	<10	80	80	160
753	40	<10	20	40
754	20	<10	20	40
778	40	160	320	160
785	160	<10	40	40

[a] Reciprocal of serum dilution.

that concentrations of 100 μg/ml of protein of *R. quintana* antigen were needed. Thus, the microtiter assay is more suitable in this instance.

The sensitivity of the EIA test assures accurate serodiagnosis of *R. quintana* infection and its simplicity should expedite delineation of the geographical distribution of trench fever. The method may be applicable to other rickettsial infections as well.

Summary. Solid-phase enzyme immunoassay (EIA) and radioimmunoprecipitation tests were developed for detection of antibodies to *Rochalimaea* (Rickettsia) *quintana* in trench fever. Both were equally sensitive, and both detected more positive sera than either passive hemagglutination or immunofluorescence tests. The advantages of EIA are the stability of label and ease and safety of use.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol 154.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 289-294 (1977)

y Effect of an Anti-oxidant, Butylated Hydroxyanisole, on the Primary *in Vitro* Immune Response (39656)

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ed hydroxyanisole (BHA) is a low weight compound used commeran anti-oxidant in foods. BHA is d generally recognized as safe by the Food and Drug Administrafoods containing BHA are, therempt from premarket clearance. In Joint Food and Agriculture Orn/World Health Organization HO) Expert Committee on Food reported that 0–0.5 mg/kg body BHA, 0–0.5 mg/kg body weight of hydroxytoluene (BHT), or a total /kg body weight of both BHA and ld be an acceptable daily intake for The average amount of BHA and sumed daily in the United States ated to be 0.2 mg/kg of each food or adults (2). The estimated intakes ght to be higher than actual intakes a calculation using manufacturing rt data would show an intake of -half of the estimated levels. Nonehe intake level is within the range d acceptable by the FAO/WHO ommittee.

port concerns the effects of BHA *vitro* murine plaque-forming cell sponse to a thymus-dependent antip red blood cells (SRBC), and a dependent antigen, *Escherichia* 7:B8 lipopolysaccharide. The reate that BHA exerts a significant effect early in the *in vitro* primary esponse at anti-oxidant concentra- may occur in the gut and liver (3– *vitro* effect and the fact that BHA resent in the gut and liver at conns used in this study warrant furly of this additive as a potential uppressant of some aspect of the response. The studies clearly show information needs to be obtained logical properties of anti-oxidants that are used as food additives.

Materials and methods. Animals. C57Bl/6 female mice, 8- to 12-weeks old, were obtained from Laboratory Supply Company, Indianapolis, Indiana or The Jackson Laboratory, Bar Harbor, Maine. Subsequently, it was shown that male C57Bl/6 mice give equivalent results. Athymic nude mice (NIH Swiss background) were supplied by the Laboratory Supply Company, Indianapolis, Indiana and were used upon delivery.

Antigens. SRBC were obtained from the Colorado Serum Company, Denver, Colorado. All SRBC used were from a single sheep, No. 446. *E. coli* 0127:B8 bacteria were treated exactly as by Johnson *et al.* (6) for the *in vitro* PFC response.

Anti-oxidant. Crystalline BHA (food grade) was obtained from ICN Pharmaceuticals, Cleveland, Ohio. Before use, BHA was solubilized in petroleum ether, washed twice with triple-distilled water, and recrystallized from petroleum ether under negative pressure. The BHA preparation was claimed to have greater than 98.5% purity as supplied, and is considered to be food grade material. Subsequent examination of the BHA preparation by thin layer and column chromatography supported this claim.

Cultures. SRBC *in vitro* PFC response were carried out as described by Mishell and Dutton (7) using 1.5×10^7 spleen cells/ml and 3×10^6 SRBC. Anti-*E. coli* 0127:B8 responses were carried out as previously described (6). The anti-oxidant was added to cultures at the time of SRBC addition in either of two ways. BHA was solubilized in mineral oil and filter sterilized, and appropriate dilutions were made in sterile mineral oil. The volume of added oil was kept constant at 100 μl, and results were compared with controls exposed to mineral oil without added BHA. BHA was also solubilized and

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ISSN 0037-9727

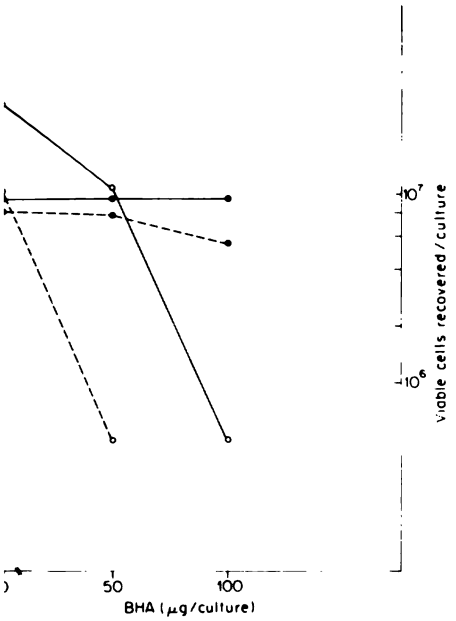
diluted in absolute ethanol (EtOH). The BHA was then added to sterile 7-mm Whatman No. 1 filter paper disks, from which the EtOH was permitted to evaporate. One disk was added per culture. Results were compared with BHA-free control disks, which were exposed to EtOH alone. Direct PFC assays were performed on microscope slides according to Golub *et al.* (8). A single lot of fetal calf serum (Gibco, Grand Island, New York) No. 640521 was used throughout the study. All PFC responses were determined on Day 5.

Enumeration and viability of cells. The number of cells in each culture was determined by counting in a hemacytometer, and the percentage of viable cells was determined by trypan blue dye exclusion.

[^{3}H]Thymidine incorporation. DNA synthesis of Mishell–Dutton cultures was determined by the addition of 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Massachusetts) in 10 μl of modified minimal essential medium (MEM) (7) to each culture for the final 18 hr of incubation. Trichloroacetic acid-insoluble material was counted in a Packard liquid scintillation counter (Model 5385). The results are based on the average of triplicate plates.

Mitogens. Concanavalin A (Con A; Nutritional Biochemical Corporation, Cleveland, Ohio), twice recrystallized, was stored at room temperature and diluted in culture medium for addition to cultures. Staphylococcal enterotoxin A (SEA) was produced by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, and its purity was estimated to be >99% by extinction coefficient (9).

Results. Effect of BHA on the in vitro PFC response to SRBC. As shown in Fig. 1, BHA inhibits the PFC response to SRBC by 91% at 50 μg/culture when added in an oil phase. BHA, though considered insoluble in water, appears to be capable of interacting with spleen cells in the aqueous phase. As shown, the viable cell recovery was not significantly reduced by levels of BHA in oil giving >90% inhibition of the PFC response. Figure 1 also demonstrates that, when administered as BHA impregnated disks, 25 μg of BHA/culture was sufficient for 90% inhibition of the PFC response.



2. Effect of removal of BHA at 4 (——) and ----) on the primary *in vitro* immune response to The direct anti-SRBC PFC/culture (○) and ells recovered/culture (●) were determined on for all cultures. BHA was added to cultures on nated cellulose disks. Disks were removed at the ed times, and the cultures were washed twice in d MEM and placed in fresh plates and growth n.

. With a 24-hr exposure of cultures, of BHA/culture was sufficient to give inhibition of the PFC response.

ct of addition of BHA at different on the PFC response. BHA-impreg- cellulose disks (25 μg of BHA/disk) added to cultures either at the same 0 time) or at 24, 48, and 72 hr after addition. Figure 3 shows that the tory effect of BHA on the PFC re- e to SRBC was decreased by delayed on. PFC inhibitions were 99, 69, and respectively, for 0 time and 24 and 48 er addition. Slight stimulation of the response was observed in cultures to BHA was added at 72 hr. Cell viabili- howed no significant differences in d versus control cultures.

ct of BHA on DNA synthesis of l/6 spleen cells. The effects of BHA, in oil or by disk, on [^{3}H]thymidine e of C57Bl/6 spleen cell cultures con- g SRBC are shown in Table I. At 50 μg/culture, BHA in oil gave 43% inhibition of [^{3}H]thymidine uptake, whereas BHA added by disc gave 88% inhibition. Removal of 100 μg of BHA/culture at 4 hr resulted in >90% inhibition of [^{3}H]thymidine uptake. Removal of 50 μg of BHA/culture at 4 hr resulted in stimulation of [^{3}H]thymidine uptake (139%). The addition of mineral oil or cellulose disks did not enhance or suppress DNA synthesis as measured by [^{3}H]thymidine uptake. In general, the data indicate that BHA exerts an inhibitory effect on DNA synthesis at higher concentrations, while having a stimulatory effect at lower concentrations. The data further suggest a complex relationship between the PFC inhibitory concentrations of BHA and its ability to inhibit DNA synthesis. In general, concentrations that inhibited the PFC response also inhibited DNA synthesis, except in the case where 50 μg of BHA was removed from culture. Here, the PFC response was inhibited (Fig. 2), whereas DNA synthesis was slightly enhanced (Table I).

Effect of BHA on the PFC response and

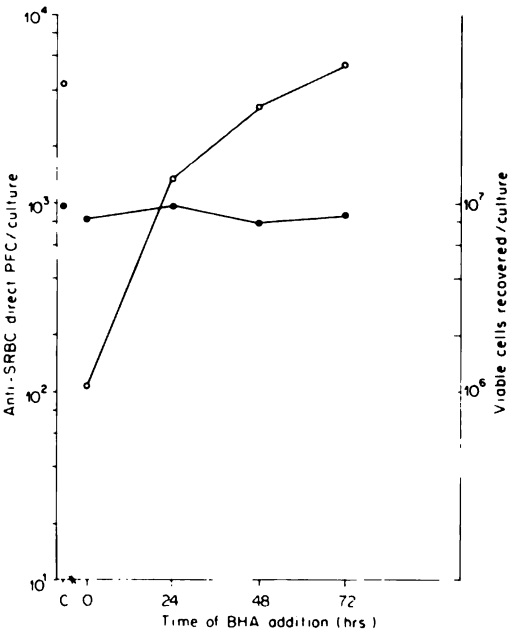


FIG. 3. Effect of addition of 25 μg of BHA (on cellulose) to mouse spleen cell cultures at the same times (0 time), and at 24, 48, and 72 hr after SRBC addition. The anti-SRBC PFC response (○——○) and viable cells recovered/culture (●——●) were determined on Day 5.

TABLE I. EFFECT OF BHA ON DNA

BHA (μg/culture)	BHA in oil[a]
200	4.5[d]
100	29.6
50	260
25	363
10	635
1	299
control[e]	455

[a] Appropriate concentrations of BHA added to culture harvested on Day 5.

[b] Appropriate concentrations of BHA added to cultı harvested on Day 4.

[c] Cultures washed free of BHA with two washes of ı medium. Cultures harvested on Day 4.

[d] Data correspond to a BHA concentration of 250 μg

[e] Contains 100 μl of BHA-free oil or EtOH-exposed

DNA synthesis of athymic nude mouse spleen cells. Table II shows the effect of BHA on the PFC response of female athymic nude mouse spleen cells to the thymus-independent antigen, *E. coli* 0127:B8. BHA, 25 μg/culture, inhibited the PFC response by approximately 53%, and higher concentrations inhibited the response by >90%. In these experiments, BHA was added to cultures on disks and remained in contact with the cells for the full 5 days of culture. No significant loss of viability occurred in any of the cultures exposed to BHA. BHA, at 25 μg/culture or more, inhibited the uptake of [^{3}H]thymidine by >99%. Interestingly, a corresponding decrease in the PFC response did not occur. Staphylococcal enterotoxin A, a T-cell mitogen (10), had no stimulatory effect on [^{3}H]thymidine uptake by athymic nude mouse spleen cells. The same concentration of enterotoxin A increased [^{3}H]thymidine uptake of C57Bl/6 cultures by 245% (data not shown). This is evidence of a lack of functional T cells in the nude mouse spleen cell cultures. High control counts in this experiment (Table II) were probably due to the large number of cells used per culture (1.5×10^7) and the presence of *E. coli O* antigen.

Effect of BHA on [^{3}H]thymidine uptake by Con A- and SEA-stimulated cultures. [^{3}H]Thymidine uptake by C57Bl/6 spleen cells, stimulated with mitogenic doses of SEA (2 μg/ml) or Con A (5 μg/ml), was

HA inhibits DNA synthesis by both mouse spleen cells (Table II) and Con nd SEA-stimulated C57Bl/6 spleen (c) BHA can inhibit the PFC response ıymic nude mouse spleen cells to *E.* 127:B8 antigen (Table II).

atment of spleen cell cultures with resulted in inhibition of both the PFC nse to SRBC (Fig. 1). and [^{3}H]thymi- uptake by the same cultures (Table I)

BHA was present for the entire ation period. BHA had to be added ltures at the same time as SRBC, later than 1 day after SRBC, for inhi- of the PFC to occur (Fig. 3). When was present for only the first 4 hr of e, a concomitant inhibition of the PFC nse and stimulatory effect on ıymidine uptake was observed (Table ıe mechanism of the PFC inhibitory of BHA appears, then, to be com- Studies are currently in progress to mine if the immunosuppressant activ- BHA is due to loss of macrophage on. PFC suppression could possibly, in cases, involve the induction of sup- ɔr-cell activity; the measurement of ɔle mediators of suppressor-cell activ- 2, 13) is in progress.

A is a commonly used food additive is readily absorbed in the intestine onjugated with glucuronic acid in the o form the o-glucuronide which is ex- l in the urine (3, 5). Studies in humans ^{14}C-labeled BHA have shown that, 2 days after ingestion, the compound is ıtirely cleared from the body (3), and ıal BHA is present for prolonged pe- after a single oral dose (4). The delay ɔretion may be due to enterohepatic ation (3–5), but, of all body tissues, ver and small intestine are probably ed to the highest levels of BHA and its ɔolic products (5). The effect of in- l BHA on the immune response of the as not been determined; however, the ɔintestinal mucosa is rich in immuno- lin-producing cells (14), and the con- nces of BHA suppressive activity ı the microenvironment of the gut are ɔwn.

ɔ widespread use of BHA and its pat- ɔf metabolism warrants further explo- ration into the mechanism of the biological effects of BHA on the immune response.

Summary. Butylated hydroxyanisole (BHA), an anti-oxidant food additive, inhibited the primary *in vitro* antibody (PFC) response of C57Bl/6 spleen cells to both a thymus-dependent antigen (SRBC) and a thymus-independent antigen (*E. coli* 0127:B8); the PFC response of athymic nude spleen cells to *E. coli* 0127:B8 was inhibited to the same degree. BHA inhibited both B- and T-lymphocyte function. BHA needed to be present only for the first 4 hr of culture to exert its inhibitory effect on the PFC response. DNA synthesis in C57Bl/6, athymic nude, and T-cell mitogen-induced C57Bl/6 spleen cell cultures was inhibited by BHA; however, short-term exposure of cultures to BHA at certain PFC inhibitory concentrations resulted in stimulation of DNA synthesis. The mechanism of the inhibitory effect of BHA on the *in vitro* PFC response is unknown, but may possibly involve activation of regulatory cell activity. Certainly, the data warrant further study of the effect of this ubiquitous food additive on the immune response.

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Received September 10, 1976. P.S.E.B.M. 197 154.

I OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 295-298 (1977)

ınce of Target Cells for Friend VIRUS (FV^P) in the Liver and Spleen of Early Postnatal Mice (39657)[1]

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in 72 hr after infection of susceptible ith a polycythemia-inducing strain of virus (FV^P), transformation of cer- mopoietic cells can be detected by ewly acquired capacity to proliferate m tumor colonies in the spleens of ally compatible recipients (1-3). Re- udies have indicated that the target this transforming effect of FV^P is a the erythroid series (4-6), although xperimentation had led other investi- to suggest that the cell may be identi- h the hemopoietic stem cell (colony- g unit, CFU$_s$) (7, 8). Our own studies experimentally manipulated hemo- cell populations, i.e., treatment with n, to suppress the hemopoietic col- ming unit (CFU$_s$) population. By nethods it is possible to dissect the ell of the virus, shown by its tumor forming capacity, from the CFU$_s$ tion. It is desirable, however, to strate, *in vivo*, the separation of the ell from the CFU$_s$ population with- rmacological intervention. This op- ty is available naturally, presented in al mice where the hemopoietic activi- the spleen and liver are increasing reasing, respectively, at this time (9, hese two tissues have been used as organs for the transforming effect of order to establish if the development FU$_s$ population corresponds with the ed availability of those target cells for virus, demonstrated by their ability tumor colonies; and correlatively, if a CFU$_s$ population leads to a de- in availability of such target cells.

rials and methods. Mice. Mice used studies were obtained from the animal production unit of Roswell Park Memorial Institute. DBA/2Cr and B6D2F1 mice were used throughout the studies. Recipient mice for irradiation studies were 3- to 5-months old, as were the B6D2F1 mice used as recipients in the tumor colony-forming unit (tCFU) assay. Postnatal mice were used at the ages indicated in the appropriate studies.

CFU assay. Cell suspensions of spleen and liver were made in phosphate-buffered saline (PBS), diluted to appropriate concentrations, and injected via a lateral tail vein into supralethally-irradiated recipient mice. X-Irradiation was done using a 250-kV Maxitron at 30 mA with 1.0-mm Al and 0.2-mm Cu filtrations and a 50-cm source-to-target distance. The normal irradiation rate was 180 rad/min as determined by a Victoreen dosimeter. A total of 900 R was administered to animals, and cell suspensions were injected into recipient mice within 2 hr after irradiation. Animals were sacrificed 9 days following irradiation and cell transplantation, and spleens were removed and fixed in Bouin's fluid. Macroscopic colonies were counted.

Virus. The Friend virus used in these studies has been previously described (11), and was passaged in DBA/2 (N-type) mice. Cell-free filtrates of spleens from leukemic mice were maintained under liquid nitrogen. Virus doses were expressed in focus-forming units, FFU (12). To achieve the high concentrations of virus used in these studies, 20% filtrates were used directly, and intraperitoneal injections were made. Stocks were titered routinely to insure uniform doses of focus-forming units/assay. At the concentration used, virus titer was not a limiting factor (See Results).

tCFU assay. Susceptibility to the disease induced by FV^P is controlled by several genes. B6D2F1 mice [F$_1$ progeny of C57Bl/

k supported in part by Grant VC-82 from the n Cancer Society and a General Research Sup- nt RR 05648 09 from the National Institutes h.

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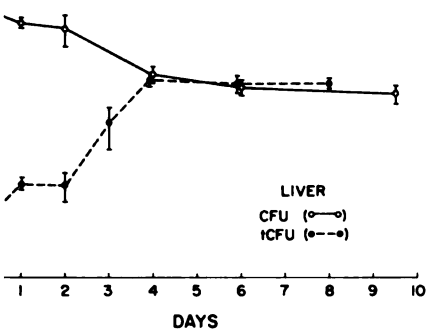
ISSN 0037-9727

6Ja (B-type) and DBA/2 (N-type) parents] are heterozygous at the Fv-1 locus (Fv-$1^{n/b}$) and are resistant to infection with the N-tropic helper virus of FV^P. Cells, transformed by FV^P from DBA/2 mice (Fv-$1^{n/n}$), will grow in spleens of B6D2F1 mice. The expression of the defective, helper-dependent SFFV of FV^P is substantially suppressed in the B6D2F1 assay animal, and the enumeration of tumor colonies is, thereby, not obscured or confused by the simultaneous induction of the macroscopically indistinguishable foci (13, 14). We routinely used a 3-day interval between infection with virus and harvest. Spleen and liver cell suspensions were prepared as before. Cell suspensions were administered via a lateral tail vein. Assay mice were sacrificed at 9 days; the spleens were removed and placed into Bouin's fluid, and macroscopically visible tumor colonies were enumerated. Data are presented for the day of virus inoculation, although cells were not obtained for tCFU assay until 3 days later. Supernatant fluids of cell suspensions used for tCFU assay were routinely assayed in DBA/2 mice for residual FFU and, except in suspensions with high numbers of tCFU, the focus-forming activity of supernatant fluids was negligible.

Results. Hemopoietic colony-forming units (CFU_s) are present in the spleens of newborn mice. Detected numbers ranged from 400–600/spleen (Fig. 1). This represented a frequency of approximately 300/10^6 spleen cells. The total number of CFU_s increases with age as the spleen expands in size to a content in excess of 2000 total CFU/spleen and a frequency approaching 1/10^5 spleen cells (Fig. 2). During this period of time, detectable target cells for Friend virus, demonstrable as tCFU, begin at very low, almost undetectable levels (Figs. 1 and 2). However, over the ensuing week to 10 days, the frequency and number of target cells for the virus increase rapidly. It is apparent, especially, that the pattern of occurrence of the tCFU target cell is substantially different from the occurrence of CFU_s, both with regard to total CFU and tCFU/spleen, as well as CFU/10^6 and tCFU/10^6 spleen cells.

This distinction is consistent with the



3. Hemopoietic spleen colony-forming units and FV^P-transformed tumor colony-forming CFU) in livers of postnatal DBA/2 mice. CFU$_s$ ined in supralethally-irradiated B6D2F1 and recipients. Distinction not made between the it strains; coefficient of correlation >0.89. The re determined in histocompatible virus-resistant 1 mice. Liver cells from FV^P-infected DBA/2 re assayed for tCFU content 3 days after intra- al inoculation of hosts with 2 × 10^4 FFU of

old DBA/2 mice were infected with 2 , 2 × 10^3, or 2 × 10^2 FFU of FV^P eritoneally in 0.1 ml of PBS. The er of tCFU in spleens was determined s later in groups of 4 B6D2F1 mice. esults of three independent studies that detectable tCFU do not differ cantly between the two higher concen- ns; $P > 0.2$ for both tCFU/10^6 spleen nd total tCFU (Table I). This shows at the virus concentration used, the pal variable determining the number ectable tCFU was the availability of priate target cells.

cussion. It is of interest that, as the l and liver, especially the spleen, es- n an equilibrium of hematopoietic ac- somewhere during the second postna- ek, the frequencies of occurrence of cells for FV^P and that for colony ng units resemble each other. This is hing that had been noticed in previous s (7, 8) and has led investigators, at to presume that the CFU$_s$ population nstitute the target cell population for ffect of the Friend virus.

superficial similarity of values for and tCFU at these times is not neces- a valid reflection of absolute compara- CFU$_s$ are only a fraction (approxi- mately 20%) of the total CFU in a population of injected cells. This fraction (f value) may be very different from the f value for tCFU, preventing a meaningful interpretation of direct, absolute quantitative determinations of tCFU and CFU$_s$.

This does not prevent the relative comparison of changing patterns of numbers of available tCFU target cells at different times in hemopoietic populations with changing CFU$_s$ content. This is conceptually different from following the growth kinetics of CFU$_s$ and tCFU within hemopoietic populations of FV^P-infected mice. The essential comparison is not necessarily whether the growth kinetics of populations of CFU$_s$ and tCFU are identical. Although interesting and important, similarity does not indicate identity. The techniques reported here evaluate the nature of the kinetics of the population that serves as a target for this transforming effect of FV^P. By fixing the time after infection when the population is assayed for tCFU content, variability in tCFU can be attributable only to variations in the number of available target cells at the time of virus inoculation.

More recent studies have indicated that the actual target cell for the virus is more closely associated with erythropoietin-responsive cells (4–6). While these are the conclusions of a number of investigators, each of these studies required experimental manipulations *in vivo* which could lead to

TABLE I. THE EFFECT OF FV^P CONCENTRATION ON THE DETECTABLE NUMBER OF TARGET CELLS FOR FV^P, DEMONSTRABLE AS tCFU IN SPLEENS OF 3-DAY-OLD DBA/2 MICE, 3 DAYS AFTER INTRAPERITONEAL INFECTION WITH VIRUS.[a]

Virus titer (FFU)[b]	tCFU in donor spleen	
	Per 10^6 spleen cells	Total per spleen
2 × 10^4	66.7 ± 10.9[c]	1398 ± 410[c]
2 × 10^3	49.8 ± 4.5	840 ± 338
2 × 10^2	0.6 ± 0.2	10 ± 0.3

[a] tCFU assay done in unirradiated B6D2F1 mice. Spleen cells (10^6) were injected into groups of four recipient mice. Data are a result of three independent studies.

[b] FV^P dose is expressed in focus-forming units (FFU)/0.1 ml injected ip.

[c] Value shown not significantly different from that determined for tCFU in spleens of mice infected with 2 × 10^3 FFU ($P > 0.2$).

some undefined disturbances in normal physiology. The studies reported here have exploited the normal variations in CFU_s in developing hemopoietic populations.

Although the neonatal mouse is immunologically immature, it is not believed that this feature contributed to the observed results. If anything, it might be that this immaturity would lead to increased susceptibility to FV^P and, thus, an increase in the availability of target cells. Therefore, it is improbable that the reduced numbers of tCFU target cells in spleens and livers at early postnatal times are a consequence of immune-mediated reductions in effective FV^P titer.

We conclude from these studies that the target cell for the Friend virus which results in autonomous growth of a cell type demonstrated by its ability to form tumor colonies in appropriate host mice is a cell which is present in hemopoietic populations normally, but is not the hemopoietic stem cell. We have not endeavored, in these studies, to correlate the population kinetics of the tCFU target cell with the erythropoietic activities of the hemopoietic populations studied.

Summary. The growth dynamics of hemopoietically active liver and spleen of neonatal mice have been used to study the association between hemopoietic stem cells (CFU_s) and a target cell for Friend virus, defined by its capacity, following transformation, for autonomous colonial growth in appropriate host mice (tumor colony forming units, tCFU). The kinetics of tCFU target cells and CFU_s are sufficiently dissimilar

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 299-303 (1977)

s of Fasting on Plasma Volume and Fluid and Sodium Exchanges in Male Rabbits (39658)[1]

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olydipsia (1-3) and increased adre-
ralocorticoid secretion (4, 5) in the
ted male rabbit have been attrib-
presumed decrease in plasma vol-
') (1, 6), a well-known stimulus to
) and adrenal mineralocorticoid se-
8); this presumption is based upon
ed natriuresis which accompanies
the male rabbit (1-3). The validity
esumption is supported by the clas-
of Gamble, Ross, and Tisdall (9)
dicated that the extracellular fluid
lecreased in the fasting human, and
vork of Bloom and Mitchell (10)
monstrated that the volume reduc-
clearly related to the urinary so-
s. The fasting-induced reduction in
ular fluid volume was subsequently
d by direct determination of
olume in the human (11-13).
jectives of these studies using adult
bits were: (i) to determine the se-
changes in PV and relevant plasma
nts during total fasting, (ii) to de-
f the time of onset of the natriuresis
l with the change in PV, (iii) to
e if the natriuresis was independent
creased urine volume by studying
etion during food and water depri-
nd (iv) to provide quantitative data
V changes which are needed for
evaluation of recently reported
in plasma concentrations of the
giotensin components in fasted rab-

ds. Thirteen adult male New Zea-
bits weighing 3.2 ± 0.1 kg were
lethods for the determination of
te content of the diet and for the
asurements of body weight, water
nd urinary volume and electrolytes
en reported (3, 5, 14). The Na^+
of the diet (Rockland Rabbit Ra-

rted by National Science Foundation Grant
014X1.

tion) was 0.01 mequiv/g dry weight. All volumes for fluid intake and urine output were corrected for evaporative losses. Blood samples were obtained during the control period (fed), after 1, 2, 3, and 4 days of total food deprivation, and after 1 day of food restoration; water was available *ad libitum* throughout. Each rabbit was cycled through this regimen two or three times following appropriate rest periods to ensure a return to normal electrolyte balance and hydration. Each rabbit was used twice for blood sampling in each cycle, except when a third sample was taken for the recovery period; samplings were always separated by at least a 3-day interval. After each control sample, the rabbits were fed for at least 3 additional days before beginning the starvation period. Pilot experiments using normal control (fed) animals indicated that the blood loss due to the sampling procedure did not alter the PV when consecutive determinations were separated by 24 hr but did reduce the hematocrit; thus, to minimize the effect of sampling volume, all PV determinations were separated by at least 3 days, and the hematocrits were not used as indices of plasma volume changes. The PV was measured by the volume distribution method using ^{131}I-labeled albumin (Albumotrope, Squibb). In addition to the control sample, four 2.0-ml serial blood samples were taken at 10-min intervals following the injection of Albumotrope. Duplicate samples were counted for gamma radiation with a Packard Model 3003 counter. Plasma protein concentration and osmolality were determined with the method of Lowry *et al.* (15) and the freezing point depression method (Fiske Osmometer), respectively. Results were statistically evaluated by means of Student's *t*-test of significance.

The daily urinary Na^+ excretion and volume were measured in an additional three adult male New Zealand rabbits when fed

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ISSN 0037-9727

and when deprived of food and water for 2 days. The Na^+ content of the diet (Teklad, Rockland) was 0.02 mequiv/g dry weight. Because of the expected low urine volume when fluid intake is zero, the bladder was catheterized, according to our previously reported procedures (14), and washed three times with 2.0 ml of tepid distilled water at the time of food and water withdrawal and at the end of each day of deprivation to ensure quantitative recovery of the excreted Na^+ within the bladder.

An additional eight New Zealand adult male rabbits were used to determine the effect of fasting on plasma glucose; four were used for blood sampling on Days 1 and 4 of deprivation, and the remaining four for sampling on Days 2 and 6 of deprivation. Plasma glucose was determined by the glucose oxidase method (GLUCOSTAT, Worthington Biochemical Corp.).

Results. The PV was significantly ($P < 0.005$) reduced from 33.4 ± 0.6 to 29.3 ± 2.1 ml/kg body weight after 1 day of food deprivation and remained at this reduced level during the remaining 3 days of deprivation ($P < 0.005$, Table I). The PV reduction was approximately 12% of the control value.

The daily urinary Na^+ excretion and volume were significantly ($P < 0.005$) increased after 1 day of food deprivation and continued to increase to reach maximal values after 3 days of deprivation ($P < 0.005$, Table I). The urinary Na^+ excretion and volume increased 12- and about 3-fold over the control value, respectively, by Day 4. Water intake also increased significantly but not until the second day of food withd water intake became maximal on th day ($P < 0.005$, Table I). The water doubled by Day 4 compared with th trol value.

Plasma osmolality was significantly 0.05) decreased from the control va 286 ± 1 to 283 ± 2 mOs*M*/liter aft day of deprivation; it progressive creased to 276 ± 8 mOs*M*/liter after of zero food intake ($P < 0.05$, Tabl

The plasma protein Na^+ and K^+ c trations were not significantly chan total food deprivation for 4 days whe pared with their respective control (Table I).

There were no statistically sign changes in the grouped absolute weights (Table I), because the weights were quite different (range: 2 kg), and the body weight of each rab not contribute to the mean of every deprivation. Furthermore, the daily weight measurement is greatly influen the balance between recent large fl take and amount of urine remaining bladder. Each animal lost weight ea of deprivation, and the average loss t 4 was 248 ± 25 g.

All parameters returned to the r control range after 1 day of *ad libitur* intake with the exception of plasma lality which remained significantly r (Table I).

When the rabbits were deprived c and water, a marked natriuresis was ir ($P < 0.005$) despite the expected red ($P < 0.05$) in urine volume (Table II

TABLE I. SEQUENTIAL CHANGES FOLLOWING CALORIC DEPRIVATION IN 13 MALE ADULT RABBIT

Parameters	Control (Fed) (17)[b]	Caloric deprivation[a] Day 1 (5)	Day 2 (5)	Day 3 (3)	Day 4 (5)	R
Plasma volume (ml/kg)	33.4 ± 0.6	29.3 ± 2.1[c]	28.4 ± 1.3[c]	29.0 ± 2[c]	29.3 ± 1[c]	34
Plasma protein (g%)	7.3 ± 0.2	7.3 ± 0.1	8.2 ± 0.7	7.2 ± 0.2	7.0 ± 0.2	7
Plasma Na^+ (mequiv/liter)	143 ± 1	145 ± 1	143 ± 1	143 ± 2	144 ± 1	1
Plasma K^+ (mequiv/liter)	4.5 ± 0	4.4 ± 0	4.1 ± 0	4.2 ± 0.1	4.7 ± 0.1	4
Plasma osmolality (mOs*M*/liter)	286 ± 1	283 ± 2[d]	281 ± 4[d]	280 ± 5[d]	276 ± 8[d]	2
Water intake (ml/kg/day)	122 ± 7	146 ± 42	173 ± 29[d]	292 ± 50[c]	251 ± 39[c]	1
Urine volume (ml/kg/day)	44 ± 6	114 ± 32[c]	101 ± 27[c]	213 ± 35[c]	166 ± 32[c]	3
Urinary Na^+ (mEq/kg/day)	0.01 ± 0	0.05 ± 0.01[c]	0.06 ± 0.01[c]	0.14 ± 0.04[c]	0.13 ± 0.05[c]	0.0
Body weight (kg)	3.2 ± 0.1	3.1 ± 0.3	3.1 ± 0.2	2.6 ± 0.2	3.2 ± 0.2	3.

[a] Each value is the mean ± SEM.

[b] Numbers of measurements in parentheses.

[c] $P < 0.005$.

[d] $P < 0.05$.

TABLE II. NATRIURESIS IN FOOD- AND WATER-DEPRIVED ADULT MALE RABBITS.[a]

Subject	n[b]	Body weight (kg)	Urinary volume (ml/kg/day)	Urinary Na^+ (mequiv/kg/day)
Control: food + water	3	3.6 ± 0.3	112 ± 17	0.02 ± 0.004
Deprived: no food and water				
Day 1	3	3.4 ± 0.4	38 ± 13[d]	0.62 ± 0.01[c]
Day 2	3	3.3 ± 0.4	14 ± 1[c]	0.82 ± 0.13[c]
Recovery: food + water	3	3.4 ± 0.3	40 ± 10[d]	0.03 ± 0.01

[a] Each value is the mean ± SEM.
[b] n, Number of rabbits.
[c] $P < 0.005$.
[d] $P < 0.05$.

control (fed) urinary Na^+ excretion and volume were greater than usual (see Table I vs Table II); this is a reflection of the greater Na^+ content of the diet used for this set of experiments.

Plasma glucose concentration was significantly reduced 24 hr after food deprivation (115 ± 4 mg%, $P < 0.02$) when compared with the control value (131 ± 3 mg%), but did not differ from the control value on Days 2, 4, and 6 following food withdrawal.

Discussion. Consonant with our previous reports (1-3), total food deprivation in the adult male rabbit induced natriuresis, polyuria, and polydipsia. Significant increases in urinary Na^+ excretion and volume occurred after 24 hr of deprivation, and a significant increase in drinking took place after 2 days; this has been the typical pattern noted previously and suggests that the polydipsia is secondary to the Na^+ and consequent PV loss. The occurrence of the natriuresis when both food and water intakes were totally restricted and with the polyuria consequently absent, provides direct evidence that the Na^+ loss in fasting animals is independent of the attendant increased urine volume and polydipsia. The polydipsia is clearly secondary to the reduced plasma volume, and it (the polydipsia), then further enhances the increased formation of a large volume of dilute urine. Adjunct support for this interpretation is provided by the observation that estrogens abolish the polyuric-polydipsic characteristics by inhibiting water ingestion but do not abolish the natriuresis (14). The primary event appears to be the increased renal loss of Na^+ when the caloric and salt deprivation of total fasting occur.

The significant changes in urinary Na^+ and volume are coincident with the PV change at the earliest time period studied, 24 hr, and this is consistent with the hypothesis of their causal role in the reduction of the PV. Thus, the presumption that a reduced PV accompanies the urinary Na^+ loss in food deprivation in the male rabbit is substantiated and is in agreement with the findings in fasting humans (9-13). The decrease in PV is much reduced or abolished when dietary sodium supplements are given to the fasting human (16, 17), which supports the view that the reduced PV of total fasting appears to be dependent upon the presence of sodium depletion (10). It is known that sodium excretion during total fasting significantly exceeds that found during low-salt plus adequate caloric intake (10, 18, 19). Similarly, the sodium-sparing action of adequate caloric intake is illustrated by the observation that feeding adult male rabbits a salt-free diet does not induce the triad of natriuresis, polyuria, and polydipsia, but caloric deprivation in the absence of a salt intake does (unpublished data); replacement of salt to the drinking water of food-deprived male rabbits abolishes the polyuric-polydipsic characteristics [(1), upublished data].

The observed decrease in PV (33.4 to 29.3 ml/kg) after 1 day of food deprivation was maintained during the entire deprivation period and was approximately 4 ml/kg or a total loss of 12-13 ml. This significant reduction in PV represents an adequate stimulus to thirst (7) and adrenal mineralocorticoid secretion (8), and it probably represents the stimulus which accounts for the enhanced fluid intake (1-3) and adrenal mineralocorticoid secretion (4, 5) reported for the food-deprived male rabbit. The increased mineralocorticoid secretion mini-

mizes the Na^+ loss in calorically deprived male rabbits (4, 5), but mineralocorticoid effectiveness under these conditions is apparently not sufficient to prevent the continued natriuresis; a similar decreased responsiveness to the Na^+-retaining action of exogenous mineralocorticoid during starvation has been noted by others [see (5, 22) for discussion and review].

The PV was maintained at a reduced value despite the continued daily natriuresis and urinary fluid loss. Since the plasma Na^+, K^+, and protein concentrations remained within the normal control range and cellular water (%) was not altered in food-deprived male rabbits (1), any movement of electrolytes out of cells was most likely accompanied by water. The maintained normal plasma [Na^+] along with the stabilized PV, albeit reduced, means that Na^+ must be moving into the plasma compartment from cells and/or the large amount of gut contents of this herbivore in order to compensate for the continued urinary Na^+ loss. The data do not provide a basis for understanding the unchanged plasma protein concentration. Failure of food deprivation to alter plasma Na^+, K^+, and Cl^- concentrations has been reported previously for the rabbit (1, 20). An inspection of Huang's data, the first report of fasting-induced polyuria-polydipsia in the rabbit, indicates that the plasma Na^+ and Cl^- concentrations did not change for approximately the first 10 days of total fasting in rabbits and, then, only gradually declined with more prolonged fasting (20).

The data do not provide an explanation for the reduced plasma osmolality and the accompanying normal Na^+, Cl^-, and protein concentrations. The decreased osmolality cannot be due to hypoglycemia, for the plasma glucose concentration was only temporarily lowered and returned to a normal level by Day 2 of food deprivation; these changes noted in glucose concentration confirm those previously reported by others (21) for the fasting rabbit. The acidosis of fasting is accompanied by an expected reduction in extracellular [HCO_3^-] (9), and replacement of the HCO_3^- by osmotically less active metabolites may contribute to the reduced osmolality; a complete plasma cation-anion analysis is required to test this possibility. In addition, the fasting-induced reduction in [HCO_3^-] may account for the renal Na^+ loss, as suggested by Chinn and co-workers (22), since an adequate interstitial [HCO_3^-] is required for renal Na^+ reabsorption (23, 24). The reduced osmolality could account for the diminished secretion of vasopressin during total fasting in the rabbit (25, 26) and apparently does not alter the ability of the contracted extracellular fluid volume to stimulate drinking (27). The reduced vasopressin secretion and the stimulation of thirst which occur in the food-deprived rabbit greatly enhance the urine flow beyond that due to the initial Na^+ loss, producing the marked polyuria.

The 12% reduction in PV can account for only a small fraction of the reported 280% and >1000% increases in plasma concentrations of angiotensinogen and angiotensin II, respectively (6). Thus, the concentration changes in the various components of the renin-angiotensin system in the fasted male rabbit are true changes and not simply the consequence of a reduced plasma volume.

It appears that the natriuresis is the initial event which triggers the cascade of events which include: a reduced PV, increased mineralocorticoid secretion, increased thirst, reduced plasma osmolality, reduced ADH secretion, and polyuria. The mechanism of the natriuresis remains unknown. The evidence cited here indicates that the reduced extracellular [HCO_3^-] and the markedly altered components of the renin-angiotensin system may possibly contribute to an intrarenal mechanism accounting for the Na^+ loss; these hypotheses should be tested experimentally.

Summary. Daily determinations of fluid exchanges, urinary Na^+ excretion, and plasma volume, Na^+, K^+, protein, and osmolality were made using 13 adult male rabbits during periods of *ad libitum* feeding, food deprivation for 4 days (water available), and 1 day of refeeding. Significant increases in urinary Na^+ excretion and volume occurred after 24 hr of deprivation, and a significant increase in drinking took place after 2 days; maximal values were obtained by Day 3 for all three parameters. The plasma volume was significantly reduced from 33.4 to 29.3 ml/kg, coincident with the changes in urinary volume and Na^+ excretion after 1 day of deprivation, and re-

mained at this reduced level. Plasma osmolality was decreased after 1 day of deprivation and continued to decline. Plasma concentrations of Na^+, K^+, and protein did not change. In a second group of rabbits deprived of food and water, a marked natriuresis was induced despite the expected reduction in urine volume. In a third group of rabbits, plasma glucose concentration was decreased after 24 hr of fasting but returned to the control level by Day 2 of fasting.

The data substantiate the assumption that the fasting-induced natriuresis and increased urine flow in the rabbit are accompanied by a reduction in plasma volume. The diuresis and natriuresis persist in fasting male rabbits with no further change in plasma volume after the first day of fasting. The natriuresis is clearly independent of the increased urine flow and fluid intake. The polydipsia is secondary to the reduction in plasma volume and enhances the increased urine flow to produce a marked polyuria.

The authors thank Mr. John Hegmann for his excellent technical assistance.

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Received September 23, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 304-309 (1977)

Hysteresis-Like Behavior of Oxygen Association-Dissociation Equilibrium Curves of Sickle Cells Determined by a New Method (39659)

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It is generally accepted that sickle cell anemia is the consequence of polymerization of mutant hemoglobin (HbS) upon deoxygenation. The mechanism of intracellular polymerization, however, is complicated by the presence of hemoglobin variants and the manner in which polymerization is brought about.

Recently, Cameron (1) observed that the oxygen affinity of sickle red blood cells (S-rbc) was greater when the equilibrium curve was determined during the process of deoxygenation by one method (2) as compared to that determined during the process of reoxygenation by a still different method (3). Rossi-Bernardi *et al.* (4) claim that the oxygenation equilibrium curves of homozygous S-rbc hemolysates after initial deoxygenation are similar to those obtained for intact normal red blood cells (rbc), whereas those of intact S-rbc show lowered oxygen affinity. Whether a change in concentration of intracellular constituents such as 2,3-diphosphoglycerate, HbF content, intracellular polymerization of HbS, pH, or other factors, is responsible for the above results needs to be shown by direct comparison of the deoxygenation and reoxygenation processes for the same S-rbc sample.

Using a newly developed instrument, the authors of the present paper have made this comparison and have determined that the initial rate of deoxygenation of S-rbc has a significant effect on the reoxygenation process.

Instrumentation. Various methods exist for the continuous determination of either oxygen association or oxygen dissociation equilibria of rbc (2-5). However, none of these methods allows the determination of both using the same sample. The instrument described herein uses less than a few drops of blood to obtain continuous curves for both oxygenation and deoxygenation equilibria starting from any desired level of oxygen saturation. Less than 5 min is required for each direction of equilibrium determination.

A simplified diagramatic sketch of this instrument is shown in Fig. 1. Whole blood is suspended as a thin liquid film on EMI micromesh in the thermostated chamber (Fig. 2). The suspended rbc within this reenforced thin film quickly establish gaseous equilibrium with the surrounding gas. Two types of gases, A and B, are mixed, saturated with water vapor, and then pumped at a near-constant net flow rate from the left of the thermostated chamber. The mixing ratio of the gases changes continually. However, the rate of change of mixing is maintained so that rbc suspended in the thin liquid film are always in equilibrium with the gas, and the rate does not exceed the response time of the Clark-type oxygen electrode. The electrode, mounted close to the rbc suspension, monitors the partial pressure of oxygen. The depolarizing current from the electrode is amplified and fed into the X axis of an X-Y recorder. Two independent wavelengths, λ_1 and λ_2, are obtained from an Aminco-Chance dual wavelength spectrophotometer. Normally, λ_1 is set at 431 nm (the Soret band of deoxygenated rbc), and λ_2 is set at 396.5 nm (the isobestic point). Spectral changes observed with the end-on photomultiplier are amplified and fed to the Y axis of the recorder.

The behavior of this instrument requires the uniformity and strength of the thin layer film. EMI nickel micromesh No. 100, 5-μm thick, provides a reproducible, sufficiently thin, liquid film, while restricting the translational motion of rbc. Thus, the number of rbc "seen" by the photomultiplier is constant, and unwanted absorbance fluctuations are reduced. The thin film of blood formed on the micromesh support can with-

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ISSN 0037-9727

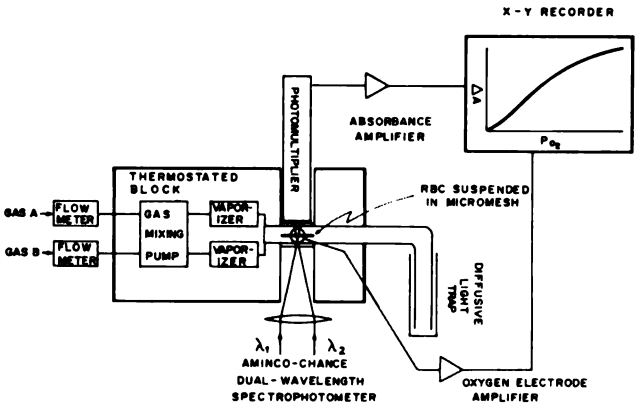

FIG. 1. A simplified diagramatic sketch of the instrument.

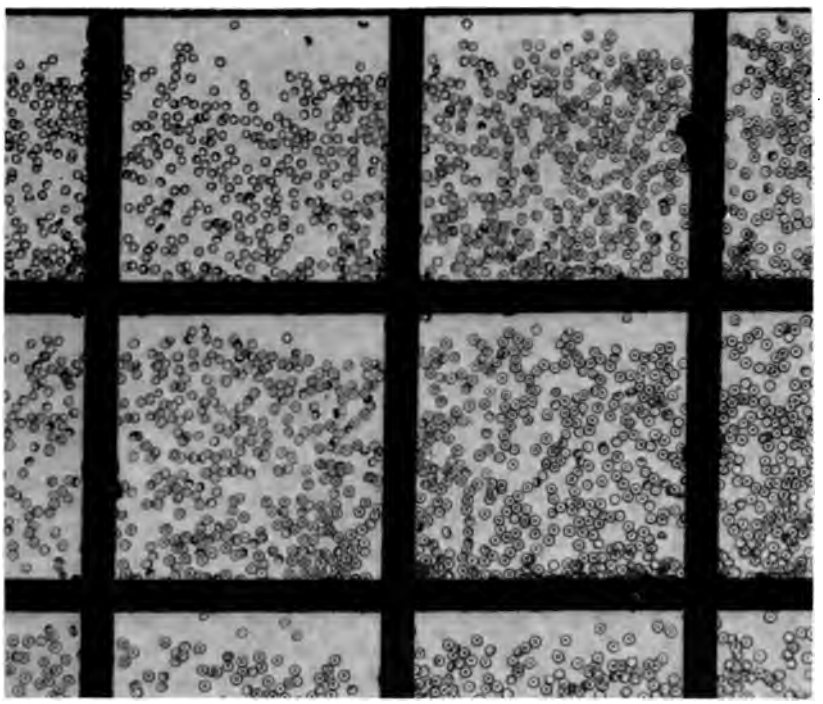

FIG. 2. Microscopic photograph of red blood cells suspended in EMI micromesh No. 100.

stand up to 20 min of constant purging by gas saturated with water vapor and flowing at the rate of 200 ml/min or more. Microscopic observation of the sample after 20 min of purging with gas did not reveal significant structural change of the rbc.

In addition to the thickness of the blood film, the response time of the Clark-type oxygen electrode is also a limiting factor for the rate of change of the gas mixing. The latter is less than 3 sec for a 90% response. A thin liquid junction lying between the electrode and the membrane is formed by pressing a drop of 3 *M* KCl hard against the polished flat electrode surface with a Teflon membrane 0.5 mil thick (DuPont or equivalent). The most rapid experimentally determined gas exchange rate with a reversible trace is 2 min in each direction. The oxygen electrode is calibrated with N_2 and 25% O_2 gases.

Test of instrument with normal blood.

Blood samples were collected from healthy subjects into heparinized Vacutainer tubes. After addition of small amounts (normally 10% by volume) of 0.9% NaCl, samples were used immediately. All experiments were carried out at 29 ± 0.5°C.

(*a*) Test for reversibility of the oxygen association-dissociation equilibrium curve: One drop of the sample was placed on the micromesh, and excess blood was removed. The mesh was placed into the reaction chamber which was preflushed with a gas mixture composed of 5% CO_2, 25% O_2, and 70% N_2 (henceforth referred to as 5% CO_2-O_2 gas). By activating the gas mixing pump, a gas mixture composed of 5% CO_2 and 95% N_2 (henceforth referred to as 5% CO_2-N_2 gas) was gradually introduced while the pumping rate of 5% CO_2-O_2 gas was reduced. In general, complete exchange of the two gases was accomplished within approximately 5 min. The net flow of the gas mixtures was maintained at ca. 200 ml/min throughout the experiment. After completion of the gas exchange, i.e., deoxygenation, the process was reversed by reintroducing 5% CO_2-O_2 gas. Several more experiments were carried out with the same sample and P_{50} values were obtained.

(b) The Bohr effect: Equilibrium curves were obtained using gas mixtures containing 2.5, 5.0, and 10.0% CO_2.

Typical oxygen-rbc equilibrium curves obtained during the process of deoxygenation (the arrow going from right to left) and that of reoxygenation (the arrow going from left to right) are shown in Fig. 3. The X axis of the recorder was calibrated with gases containing 25 and 0% O_2 and was converted to read Po_2 in mmHg after correcting for vapor pressure at that temperature and atmospheric pressure. The Y axis of the recorder monitored oxygen saturation of rbc's, assuming the rbc's were fully saturated in gas containing 25% O_2 and deoxygenated in gas containing 0% O_2. As can be seen in Fig. 3, the equilibrium curves obtained during the process of deoxygenation and reoxygenation are nearly identical, confirming that equilibrium was maintained at all times. The half-saturation point, P_{50}, thus obtained, is 19.5 mmHg and is close to that tabulated elsewhere (6) at this temperature and CO_2 content.

Figure 4 shows the saturation curves obtained during the process of deoxygenation using a gas mixture containing 2.5, 5.0, or 10% CO_2. Although not shown, the reversibility of the curves (for both deoxygenation and reoxygenation processes) under present conditions has been confirmed. The fact that all three curves do not have an identical total absorbance change is primarily due to the differences in the populations of rbc's seen by the photomultiplier tube. The average P_{50} for gas mixtures containing 2.5, 5.0, and 10.0% CO_2 are 16.8, 19.3, and 24.5 mmHg, respectively, and are summarized in Table I. After correcting for temperature and Pco_2, these values are similar to those reported elsewhere (6).

Experiments with S-rbc. S-rbc were obtained from a homozygous sickle cell anemia patient (HbS 87.0% and HbF 10.5%), and the sample was used within 4 hr after collection of the blood in heparinized Vacutainers (equivalent to two drops of sodium heparin in 10 ml of blood). Homozygous sickle cell blood from two other individuals was also tested. However, due to the varia-

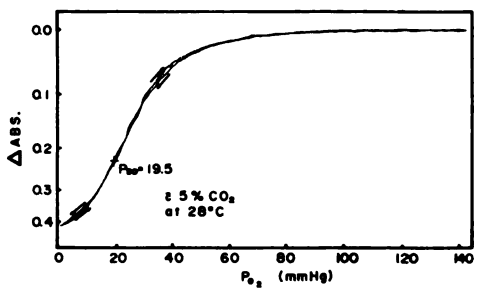

FIG. 3. Oxygen association-dissociation equilibrium curves of whole blood at 28° in 5.0% CO_2. The direction of the arrows indicates the change of partial pressure of oxygen.

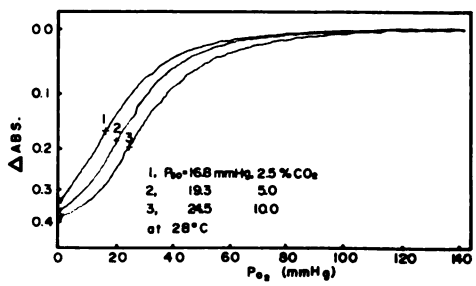

FIG. 4. The oxygen dissociation equilibrium curves of whole blood in 2.5, 5.0, and 10.0% CO_2 at 28°.

E I. HALF OXYGEN SATURATION PRESSURE
E BLOOD AT VARIOUS CO_2 LEVELS AT 28°C.

b) P_{CO_2} (mmHg)	$t_{1/2}$ (mmHg)[a]	P_{50} (mmHg)[b]
17.9	13.6	16.8[c]
35.8	16.7	19.3 ± 1.2[d]
71.6	21.0	24.5[c]

(6).
experiment.
age of three.
n six runs.

f hemoglobin composition in each
ıal blood sample, no quantitative
ison among the subjects will be at-
d at this time. The individuals tested
been on chronic drug therapy or had
dical treatment during the last 24 hr.
ıcing of the curve in the direction of
leoxygenation or reoxygenation took
imately 10 min. Although not
temporary maintenance at any par-
partial pressure of oxygen did not
significant change in absorbance,
ng that the S-rbc and the environ-
gases were in equilibrium.

lts. Typical oxygen association–disso-
equilibrium curves of S-rbc are
in Fig. 5. The curves obtained during
enation are shown in continuous
nd those obtained during reoxygena-
: shown in broken lines. The heavy
present experiments in 10.0% CO_2
: thin lines represent those in 5%
ıs can be seen in each CO_2 system,
ve obtained at the time of reoxygena-
shifted to the right of those obtained
deoxygenation, thereby displaying
sis-like phenomena. Similar experi-
results for two other homozygous
s (F. S. and E. S.) are shown in Fig.
an be seen that, in both samples,
sis-like phenomena persist.

r replotting the Fig. 5 results as Hill
Fig. 7), the P_{50} and Hill constant, n
, of each curve are obtained, and the
are summarized in Table II. The P_{50}
ıd depends upon 2,3-diphosphogly-
pH, P_{CO_2}, temperature, and the
t of hemoglobin variants (11). Ac-
ly, P_{50} values reported here are not
rily representative of S-rbc. In addi-
the hysteresis-like phenomena de-
, it can be seen that the Hill constants
or both CO_2 systems are less at the

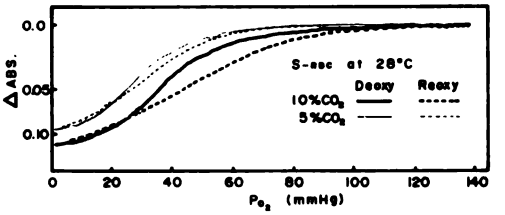

FIG. 5. The oxygen association–dissociation equilibrium of sickle cells at 28°. The curves obtained during deoxygenation are shown in continuous lines, and those obtained during reoxygenation are shown in broken lines. The heavy lines represent experiments in 10.0% CO_2, and thin lines are for 5.0% CO_2.

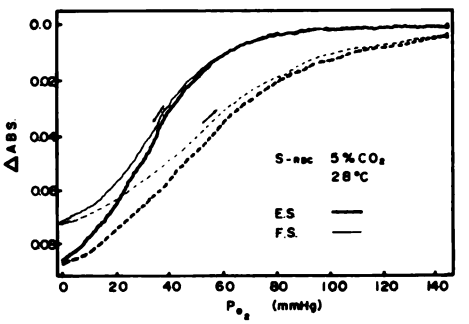

FIG. 6. The oxygen association–dissociation equilibrium curves of sickle cells from two other homozygous patients at 28° in 5% CO_2.

time of reoxygenation than are those of deoxygenation. The change in the constant was greater for the 10% CO_2 system. As has been observed by others (7, 8), the Hill constant at P_{50} is relatively small for the equilibrium curve at the time of reoxygenation, but increases considerably as the degree of oxygen saturation increases. This apparent increase in cooperativity in the higher region of oxygenation may be related to the depolymerization of HbS.

Discussion. It has been observed that the oxygen dissociation equilibrium curve of dilute HbS in solution resembles that of normal hemoglobin (HbA) in solution (9, 10). The oxygen dissociation equilibrium curve of S-rbc, on the other hand, is shifted to the right of that of normal rbc. While neither phenomenon has been adequately explained, the intracellular level of 2,3-DPG, the hemoglobin concentration, the level of HbF, the pH, the degree of gelation, and the properties of cellular membranes have been considered responsible for the shift in the curve of S-rbc. Technical difficulties,

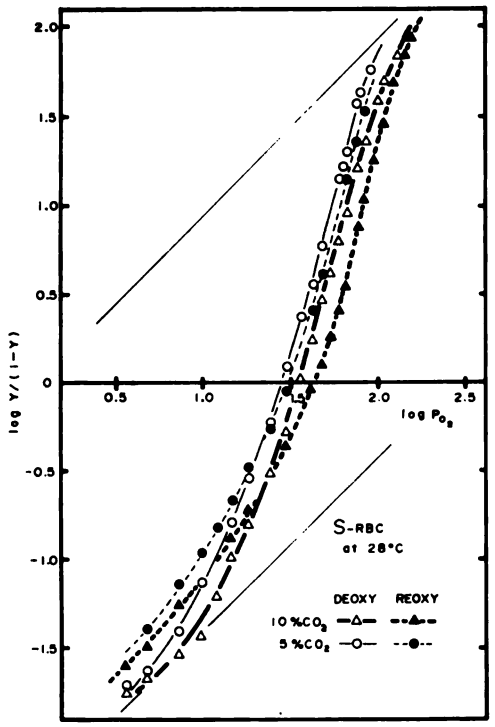

FIG. 7. Hill plot of Fig. 5.

TABLE II. OXYGEN AFFINITY (P_{50}) AND HILL'S CONSTANT (n) OF HOMOZYGOUS SICKLE CELLS DETERMINED AT 28°C.[a]

CO_2 (%)	P_{50} (mmHg)		n (at P_{50})	
	Deoxy	Reoxy	Deoxy	Reoxy
5.0	28.2	31.3	3.0	2.7
10.0	31.8	43.7	3.25	2.5

[a] Average of three runs.

however, prevented the investigators from comparing the oxygen equilibrium curves at the time of deoxygenation and reoxygenation using the same S-rbc. An experiment performed within the period that causes little or no intracellular chemical change will provide evidence for the difference observed between the two equilibrium curves being attributable to a change in physical status. To this end it is important to note the change of physical status observed in HbS solution.

According to Eaton *et al.* (12), the critical temperature of the gelation of deoxygenated HbS in solution is dependent upon the rate of temperature change. Specifically, the critical temperature is lower if the sample is warmed slowly. This observation implies that the thermal motion of deoxygenated HbS becomes the rate-limiting step when the solution temperature is rapidly changed. This thermal motion of HbS will directly affect the number and size of polymer crystals serving as nuclei of crystallization. On the other hand, upon deoxygenation of S-rbc, there occurs a conformational transition of HbS from R to T state, and gelation (or polymerization) will be initiated. It is then possible that the rate of deoxgyenation can also affect the number and size of seed nuclei formed intracellularly. If sickling is caused by a rapid deoxygenation process, the polymers formed are less ordered, or inhomogeneous. Josephs *et al.* (13) also observed at least two types of helical fibers. Curve 1 in Fig. 8 was obtained at the time of reoxygenation of S-rbc which had previously been quickly deoxygenated by insertion of the sample into the deoxygenation chamber. Immediately after completion of reoxygenation, curve 2 was obtained by slowly deoxygenating the same sample in the usual manner. Curve 3 was obtained by slowly reoxygenating the sample a second time. Curves 1 and 2 are similar in that they are both sigmoidal and have approximately the same oxygen affinity, whereas only curve 3 shows an apparent reduction in the sigmoidal shape and a decrease in oxygen affinity.

Based on these observations we suggest that: During deoxygenation, sickle cell hemoglobin interacts as independent molecules until a certain degree of deoxygenation, at

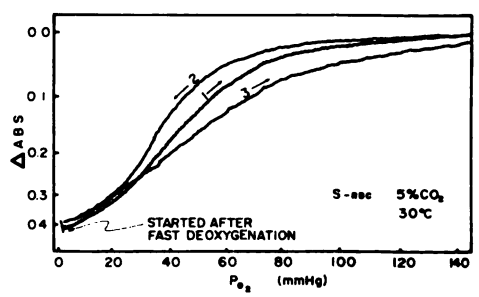

FIG. 8. The first oxygen association equilibrium curve (1) was obtained by placing the sample directly into the previously deoxygenated chamber, thereby deoxygenating the sample quickly. After completely reoxygenating the sample, it was slowly deoxygenated (curve 2) and reoxygenated slowly (curve 3). Reduced sigmoidness is observed only in curve 3.

which point the nucleation of the molecule takes place. The molecule will be further polymerized as the deoxygenation continues to completion. When reoxygenation is initiated, the polymeric form, for which oxygen affinity is lower than for the nonpolymeric form, will be maintained despite a considerably higher partial pressure of oxygen, thereby presenting an apparent shift of the equilibrium curve to the right. On the other hand, polymers formed during rapid deoxygenation accomplish depolymerization at a lower partial pressure of oxygen, hence, only a small shift of the equilibrium curve is observed. Although these conclusions are only qualitative and need further study, it can be assumed that the hysteresis-like behavior of the oxygen association–dissociation curves at the temperatures studied results from the formation of specific intracellular polymers during the time of deoxygenation and that variation in polymer structure occurs in varying rates of deoxygenation, causing variation in the reoxygenation curve.

Summary. An instrument to determine the oxygen association–dissociation equilibrium of whole blood has been developed. Only one drop of blood sample is required to trace automatically the oxygen–red blood cell equilibrium curve in the direction of deoxygenation and reoxygenation within a total of less than 10 min. The whole blood is suspended in the form of a thin liquid film reenforced by a micromesh. The partial pressure of oxygen is changed continuously with a gas-mixing pump while simultaneously being recorded by polarographic oxygen electrodes. The spectral change of red blood cells is recorded by a dual-beam spectrophotometer.

Using this instrument it is shown that the oxygen equilibrium curve of sickle cells obtained during deoxygenation is shifted to a higher oxygen affinity as compared to those obtained during reoxygenation. This hysteresis-like behavior of equilibrium curves is also reflected in the Hill constant, n, which is greater for deoxygenation than for reoxygenation. On the other hand, if deoxygenation is completed rapidly, the sigmoidness of the reoxygenation equilibrium curve and its oxygen affinity are greater than that obtained after slow deoxygenation. Hysteresis-like behavior is the postulated consequence of intracellular polymerization of sickle hemoglobin.

The authors wish to thank Dr. George Brewer from the University of Michigan for helpful comments, and to acknowledge the cooperation kindly extended to them by the Comprehensive Sickle Center of Wayne State University in supplying blood samples and the support of a WSU Faculty Research Award, and a grant from NHLI.

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Received June 28, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 310-313 (1977)

The Effect of β-Aminopropionitrile (BAPN) on Bone Mineralization (39660)[1]

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There is evidence that collagen soluble in neutral salt solutions represents recently deposited collagen, and that more mature collagen is insoluble by virtue of intermolecular crosslinks (1). In bone, about 98% of collagen is insoluble (2). Very little increase has been found in the amount of collagen extracted by neutral salt solution after demineralization in EDTA (3). These findings suggest that the majority of soluble collagen is located in unmineralized osteoid which consists of recently deposited collagen, and that the mineral phase is located in insoluble collagen. Moreover, it has been suggested that the formation of intermolecular crosslinks in collagen may precede mineral deposition in bone matrix and may, thus, be a prerequisite for appropriate bone matrix mineralization (4).

If collagen crosslinking of bone matrix is somehow involved in bone mineralization, one would expect to find impairment of bone mineralization in response to lathyrogens, such as β-aminopropionitrile (BAPN), which are strong inhibitors of lysyl oxidase and, thus, of collagen crosslinking (5). The present study was undertaken to evaluate the effect of BAPN treatment on bone mineralization in rats.

Material and methods. Thirty growing male Holtzman rats weighing 66.2 ± 1.9 g (SD) were divided into control and experimental groups. The rats were housed separately in wire cages, and the control group was pair-fed a semisynthetic diet containing 0.6% calcium and 0.6% phosphorus, dry weight (6). For the experimental group, β-aminopropionitrile fumarate, 0.1% dry weight, was added to this diet. The animals were treated for 15 days. Five days prior to sacrifice, they were injected ip with 20 mg of tetracycline per kg body weight.

Just prior to sacrifice, blood was obtained by cardiac puncture under light ether anaesthesia. The animals were then killed by exsanguination. The tibiae were removed and stored in gauze moistened with a 10% buffered formalin solution, pH 7.4. This treatment has a negligible effect on calcium and phosphorus concentrations in young as well as mature bone, as determined by the electron microprobe (unpublished observation). The left femora were removed and stored at −20° for subsequent bone ash analyses.

Serum analyses. Serum calcium was analyzed by atomic absorption spectrophotometry (7), and phosphorus by a conventional Technicon autoanalyzer technique.

Bone ash analyses. The left femora were thawed, dissected free of soft tissue, and sawed at each end to secure diaphyseal bone (middle third) from which the bone marrow was removed by a jet of air. After rinsing in distilled water, the diaphyses were dehydrated and defatted in acetone, changed daily for 3 days. Subsequently, the bones were air dried at room temperature, weighed, and then ashed at 600° for 16 hr. The ashed bones were then cooled for 2 hr and weighed. The ash was analyzed for calcium (7) and phosphorus content.

Bone measurements. From each tibia, two 60-μm-thick sections were sawed immediately proximal to the tibio-fibular synostosis with a Gillings-Hamco thin sectioning machine (Hamco Machine, Inc., Rochester, N.Y.). The bone specimens were mounted in a goniometer to facilitate sawing sections perpendicular to the long axis of the tibial diaphysis. The sections were then hand

[1] This work was supported in part by NIH grants DE-02600 and HD-04872. Dr. D. Baylink (MRIS 0483) is recipient of RCDA award DE-19108. Dr. Rosenquist received research and training support from the University of Umea and the Swedish Medical Research Council.

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ISSN 0037-9727

ınd to a final thickness of about 25 μm. sections were labeled *in vitro* with tetra- ine by placing them for 1 min in a 0.2% eous solution of Achromycin.[4] After ng in distilled water, the distal sections e mounted unstained in Abopon (Val-Co., Brooklyn, N. Y.) and the proximal ions were stained with nuclear fast red, ydrated in acetone, cleared in xylene, mounted in Fluormount (E. Gurr Ltd., don S. W. 14, England). The unstained ions were used for measurements of to- one area, periosteal surface length, area mpassed by the periosteal tetracycline l, the length of this label (given 5 days r to sacrifice), and width of the *in vitro* tracycline label. The stained sections were l for osteoid width measurements.

hese measurements were performed us- a model 1010A Grafacon tablet (Com- etics Inc., Monroeville, Penn.) inter- d with a computer (PDP-8, Digital ipment Co., Manor, Mass.). The calcu- ns of the bone parameters from the ve measurements have been described er (8) and are only defined below.

) Periosteal bone formation rate (mm^3/ is the amount of bone or matrix formed ne periosteal surface per day. Dividing rate by the length of the forming surface s the bone or matrix apposition rate /day).

) Periosteal osteoid maturation rate hr) is a measure of the onset of minerali- n. The time elapsing between the depo- n of osteoid and the onset of mineraliza- in this osteoid can be calculated by ling the width of the osteoid by the ix apposition rate. If one assumes the oid to be 0% mature when first depos- and 100% mature at the onset of miner- tion, the osteoid maturation rate can be ulated simply by dividing 100% by the ve time.

i) Periosteal initial mineralization rate f maximum/hr): It has been shown that cycline is only incorporated into bone has 20% or less of its maximum mineral entration (8). The time required to h 20% of maximum mineralization is rmined by converting the width of the *in vitro* tetracycline band to time, using the bone apposition rate. Dividing 20% (mineral concentration) by this time gives the rate. The initial mineralization rate and the osteoid maturation rate measure two different aspects of mineralization. The initial mineralization rate is the rate at which mineral concentration increases (up to 20% of maximum) once mineralization is initiated at the mineralizing front. In contrast, the osteoid maturation rate is a measure of the onset of mineralization, which is a function of the time elapsing between the deposition of osteoid matrix and the onset of mineralization in this matrix.

Results. Two of the 15 BAPN-treated rats had final weights of 85 and 75 g, compared with 135.9 ± 15.6 g (2 SD) for the other BAPN-treated rats, and were therefore excluded. In the remaining test animals, physical activity and appearance were similar to the control rats throughout the experiment. As indicated by the final body weight (Table I) and total cross-section area (bone and medullary area) of the tibial diaphysis (Table II), growth was similar in the two groups.

As can be seen in Table II, there was a significant decrease of 31% ($P < 0.001$) in the initial mineralization rate in the BAPN group, compared with the pair-fed control rats. However, BAPN treatment did not alter the onset of mineralization as indicated by the normal osteoid maturation rate. The ash weight of the diaphyseal (cortical) bone was significantly reduced by 5% ($P < 0.001$), whereas the ratio of Ca/P in bone ash was unchanged by BAPN treatment.

Discussion. Our results showed that, in rats, BAPN treatment caused a highly significant 31% decrease in the initial mineralization rate of diaphyseal bone. However, BAPN treatment did not inhibit the osteoid maturation rate and, thus, did not inhibit the onset of mineralization. In past studies, we consistently found that a change in mineralization rate was accompanied by a similar change in the onset of mineralization (8–10). Therefore, it is noteworthy that BAPN treatment impaired only one aspect of the mineralization process, which suggests that these two aspects of mineralization may be, to some extent, controlled independently.

Consistent with impaired mineralization

Generously supplied by Lederle Laboratory Divi- American Cyanamide Co., Pearl River, N.Y.

TABLE I. BODY WEIGHT, SERUM CONCENTRATIONS, AND BONE ASH WEIGHT (CORTICAL DIAPHYSEAL BONE) IN CONTROL AND BAPN-TREATED RATS.[a]

Paramater	Control (n = 15)	BAPN-treated (n = 13)	P[b]
Final body weight (g)	133.6 ± 9.6[c]	135.9 ± 7.5	NS[d]
Serum Ca (mg/100 ml)	11.4 ± 0.3	11.2 ± 0.3	NS
Serum P (mg/100 ml)	8.8 ± 0.5	8.6 ± 0.5	NS
Bone ash weight (% of dry weight)	60.6 ± 1.3	57.3 ± 2.5	<0.001
Ca/P of bone ash	1.69 ± 0.06	1.67 ± 0.05	NS

[a] Diet: 0.1% BAPN.
[b] Probability estimated by Student's t-test.
[c] Mean ± SD.
[d] Not statistically significant at the 0.05 probability level.

TABLE II. PERIOSTEAL FORMATION AND MINERALIZATION IN CORTICAL BONE OF CONTROL AND BAPN-TREATED RATS.[a]

Parameter	Control (n = 15)	BAPN-treated (n = 13)	P[b]
Total cross-section area (mm^2)	3.197 ± 0.245[c]	3.236 ± 0.165	NS[d]
Periosteal bone formation rate (mm^3/day)	0.067 ± 0.006	0.065 ± 0.005	NS
Periosteal bone apposition rate (μm/day)	10.3 ± 0.8	10.0 ± 0.9	NS
Periosteal initial mineralization rate (%/hr)	2.6 ± 0.4	1.8 ± 0.3	<0.001
Periosteal osteoid width (μm)	7.4 ± 1.4	6.7 ± 0.9	NS
Periosteal osteoid maturation rate (%/hr)	5.9 ± 1.5	6.3 ± 1.0	NS

[a] Diet: 0.1% BAPN.
[b] Probability estimated by Student's t-test.
[c] Mean ± SD.
[d] Not statistically significant at the 0.05 probability level.

was a 5% decrease in the percentage of bone ash in diaphyseal bone of the BAPN-treated group (Table I). Although a decrease in the percentage of bone ash has been previously observed in response to BAPN treatment, it is not clear whether or not the bone samples contained epiphyseal cartilage and/or marrow (11).

It seems doubtful, however, that a 31% decrease in the mineralization rate could have accounted for a 5% decrease in the percentage of total bone ash since, in normal diaphyseal bone from rats of the age and size used in this study, the volume of bone undergoing mineralization is only about 10% of the total bone volume (9, 13). Moreover, if, as seems likely, the decrease in bone ash was limited to the volume of lathyritic bone formed during BAPN treatment, which was about 36% of the bone sample (9, 13), the decrement in the percentage of ash in the lathyritic bone would have been much greater than the 5% decrease observed with the total diaphyseal bone sample. Thus, there may have been a decrease in maximum mineralization as well as a decrease in mineralization rate. Definitive assessment of this possibility will require electron microprobe measurements of calcium and phosphorus concentrations in lathyritic bone.

This study does not establish the mechanism whereby BAPN treatment inhibits the process of bone mineralization. Because BAPN treatment at the dose used in the present study increases the fraction of soluble collagen in bone and, thus, presumably decreases collagen crosslinking, it is possible that the latter is involved in the observed mineralization defect. Alternatively, it is possible that impaired bone mineralization and impaired bone collagen crosslinking are independent effects of BAPN treatment. If impaired collagen crosslinking inhibits the process of bone mineralization, this mechanism could be operative in vitamin D deficiency since, in bones from vitamin D-deficient animals, there is some evidence of decreased lysyl oxidase activity (15) and impaired collagen crosslinking (16).

Summary. The process of bone mineralization in the tibial diaphysis, in response to treatment with a lathyrogen, β-aminopropionitrile (BAPN), at a dose known to inhibit collagen crosslinking, was studied in growing rats. BAPN treatment impaired the

initial rate of mineralization but not its onset. In addition, in the BAPN treated group, there was a 5% decrease in diaphyseal ash weight, a decrement estimated to be greater than expected from the decreased rate of mineralization. This suggests that the maximal level of mineral deposition was also decreased as a result of BAPN treatment.

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Received March 23, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 314-317 (1977)

Immunization by the Intestinal Route of C3H-Mice against C3H-L-Ascites Tumor Cells (39661)

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Introduction. In a report presented in 1880 to the Academie des Sciences in Paris, the idea of oral vaccination as a means of conferring immunity was suggested by Pasteur, Chamberland, and Roux (1). These eminent authors pointed out that chickens not only survived but were rendered immune after ingesting food saturated with the organism of chicken cholera. In 1891, Ehrlich (2) demonstrated antitoxin in the blood of mice immunized by feeding them on cakes containing vegetable poisons such as ricin and abrin. The presence of specific antibody activity in intestinal secretions was demonstrated in 1922 by Davies (3) who examined the feces of patients suffering from dysentery. In 1957, Lund (4) reported that rats treated with intracecal injections of Yoshida's ascites tumor cells developed complement-fixing antibodies and resistance to secondary challenge with this tumor. Intestinal vaccination with viruses and bacteria has since been confirmed in numerous studies. The participation of the intestinal lymphoid system in the natural defense against cancer is not clear. However, in view of the fact that the intestinal wall is one of the major sites of lymphocyte production, this question deserves more attention. Studies of this type may provide us with a better understanding of variation in the immunoresistance to cancer. The present investigation confirms the isoimmunizing properties of malignant syngeneic tumor cells inoculated into the lumen of cecum in inbred mice.

Materials and methods. Seven- to eight-week-old inbred mice of the C3H/Fib strain were used for this work. The animals were kept under conventional conditions with up to 10 mice in each cage. The malignant cell material was derived from cultures of C3H mouse lung fibroblasts which had undergone apparently spontaneous malignant conversion and transformation during propagation *in vitro* (5). From these cells an ascites tumor (C3H-L 1/a) was established which grew equally well *in vivo* and *in vitro*. On intraperitoneal inoculation, TD_{50} was 2×10^6 cells. On subcutaneous inoculation, small tumors were produced which regressed within 2–4 weeks. After a small laparotomy, 10^7 cells in a volume of 0.1 ml were inoculated into the cecal lumen of ether-anesthesized mice using injection needle 27 Gx1. After the injection, the cecum was washed in isotonic saline before replacement. Specific antibodies were demonstrated by the indirect immunofluorescence technique, using live C3H-L 1/a cells as target cells and serial dilutions of mouse sera obtained by bleeding from the retroorbital sinus. The sera were stored at −70°. Staining was done with fluorescein-conjugated pig anti-mouse globulin purchased from Hyland. The fluorescent index was calculated as described by Klein and Klein (6). Tumor neutralization tests were made with lymph node cells, splenocytes, and antisera obtained from donors immunized by intracecal inoculation of 10^7 C3H-L 1/a cells. Lymph nodes and spleens were removed under sterile precautions and single-cell suspensions were prepared in phosphatebuffered saline (PBS) after gentle homogenization of the lymphoid organs in a glass homogenizer with a loose fitting pestle. C3H-L 1/a tumor cells were added to the lymphocyte suspension at a lymphocyte/tumor cell ratio (L/T) of 7/1 or 15/1, and the tumorigenicity of the mixed cell population was finally tested by intraperitoneal injection into nonsensitized C3H recipients at a tumor cell dose of 10^7 per animal. In similar experiments C3H-L 1/a cells were suspended in undiluted antisera at a concentra-

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ISSN 0037-9727

)f 10^7 cells per 0.4 ml. No complement added. Indirect immunofluorescence on these suspensions showed 100% ion. The experiments were discontin- 00 days after challenge.

ults. The protective effect of lumenal :ecal (ic) inoculation of 10^7 malignant ·L 1/a cells against secondary intraperi- il challenge 8–26 days later with the malignant cells is shown in Table I. As)e seen, a total of 72 (92%) of the 78 inized mice survived challenge with 10^7 ·L 1/a cells. The survival rate in the ol group was only 24% (5/21). Table I shows that neither frozen and thawed nor supernatant fluid from such a fro- nd thawed cell suspension gave rise to otective effect when inoculated ic st a secondary ip challenge with live Table II shows the results of the indi- immunofluorescence tests carried out pooled sera from eight mice inocu- once or twice with 10^7 live C3H-L 1/a ic. The animals were sacrified 9 days the last ic inoculation. It appears that at a dilution of 1:16, the fluorescent ; was above 0.3. The indirect immuno- escence test did not show new antibod- ɔ membrane antigens on C3H-L 1/a in a sera from mice immunized ic with n and thawed cells. Neither did this iique give positive results when using from mice immunized ic or sc with :natant fluid from frozen and thawed

'ABLE I. PROTECTIVE EFFECT OF LUMENAL RACECAL (IC) INOCULATION OF MICE WITH 10^7 C3H-L 1/a CELLS AGAINST A SECONDARY LLENGE WITH THE SAME CELLS (IP) IN C3H/Fib MICE.

between ic and ip lations of live cells	Mice dead with tumor/total mice		
	Male	Female	Sum
8	1/10	0/8	1/18
15	0/10	2/10	2/20
26	0/10	1/10	1/20
26 and 18	2/10	0/10	2/20
of immunized	3/40	3/38	6/78
ɜ			
ımunized controls	8/11	8/10	16/21
mmunized ic with frozen and ved cells × 5			8/9
immunized ic with supernatant fluid from a frozen and ved cell suspension			10/13

TABLE II. INDIRECT IMMUNOFLUORESCENCE TEST.[a]

Serum dilution	Fluorescent index[b]	
	Immunizing injection (number)	
	1	2
1:1	0.99	0.96
1:2	0.94	0.96
1:4	0.76	0.84
1:8	0.48	0.71
1:16	0.35	0.51
1:32	0.10	0.24

[a] Target cells: C3H-L 1/a. Sera: C3H mice inoculated once or twice ic with 10^7 C3H-L 1/a cells and bled 9 days after the last injection.

[b] (a − b)/a, where a = the percentage of fluorescent negative cells treated with normal control serum, and b = the percentage of fluorescent negative cells treated with antiserum.

cell-suspension. Tumor neutralization tests were made with sera and lymphocytes from C3H mice immunized twice by the ic inoculation of 10^7 C3H-L 1/a cells 2 and 3 weeks before sacrifice. In animals reimmunized after 1 week, the cecum and the abdominal cavity were examined but no indication of tumors in or outside the cecum were seen. The results of three experiments are shown in Table III from which it appears that the antisera were without any tumor-neutralizing effect. Lymph node cells had no statistically significant effect, whereas splenocytes showed a pronounced inhibitory effect. In some of these experiments it was observed that the mice developed ascites the first 7 or 8 days, and to the same degree whether the splenocytes were transferred or not. After this period, however, the ascites suddenly disappeared in those groups which had received sensitized splenocytes mixed with the malignant cells. Table III also shows that preincubation of the tumor cells with specific antiserum for 30–60 min at 37° did not block the neutralizing effect of sensitized splenocytes.

Discussion. By the ic inoculation of Yoshida's ascites tumor cells, Lund (4) induced immunity in rats against a secondary ip challenge with the same tumor cell line. This author also demonstrated specific complement fixing antibodies in ic immunized rats (4, 7). These experiments were carried out in an allogeneic system. In the present

TABLE III. TUMOR NEUTRALIZATION TEST.[a]

	Group							
	I		II[b]		III	IV	V	VI
	Sensitized lymph node cells		Sensitized splenocytes			Antiserum + sensitized splenocytes	Nonsensitized splenocytes	
Experiment	7:1[c]	15:1	7:1	15:1	Antiserum	15:1	15:1	Nihil
1	8/10[d]			1/10	7/8	0/10		9/10
	24 ± 3.0[e]			73	22 ± 2.2			23 ± 2.6
2	4/10	3/5	1/10	0/10	4/10	1/10		8/10
	24 ± 2.5	18 ± 3.0	23		25 ± 3.5	25		23 ± 2.6
3	5/6	11/17	4/18	2/23	16/19		14/18	17/20
	21 ± 3.8	23 ± 4.5	23 ± 1.8	20-2.0	20 ± 1.4		23 ± 2.4	23 ± 3.2
Sum of three experiments (percentage dead)	17/26	14/22	5/28	3/43	27/37	1/20	14/18	34/40
	65	64	18	7	73	5	78	85

[a] C3H/Fib-mice were immunized twice by ic inoculation of 10^7 C3H-L 1/a cells and used as donors. Serum, lymph node cells, and splenocytes from these donors were mixed with C3H-L 1/a cells, and the mixtures were transplanted ip to untreated C3H-Fib-mice. In one group (V) splenocytes from nonimmunized donors were used.

[b] Groups of II and IV significantly differ from the other groups at the 99% level.

[c] Lymphocyte/tumor cell ratio.

[d] Number of dead mice/total mice.

[e] Time of survival after challenge expressed in days.

study, syngeneic C3H-L 1/a tumor cells showed isoimmunizing properties in inbred C3H mice after ic inoculation of live malignant cells, but not after ic inoculation of frozen and thawed cells. The isoimmunizing effect was demonstrated by resistance to secondary ip challenge, the presence of specific antibodies as evidenced by a positive indirect immunofluorescence test, and by the protective effect of sensitized splenocytes in a tumor-neutralization test. The fact that frozen and thawed cells inoculated in the cecal lumen did not give rise to a protective effect against a later challenge indicates that the inoculated cells have to be viable and that macromolecules which are supposed to be liberated from the frozen cells are without any protective effect. Neither did Kieler *et al.* (5) obtain any evidence which supported the suspicion that antigenic properties of C3H-L 1/a-cells are due to virus.

From the observations made so far, it is not clear whether immune stimulation depends on the presence of intact tumor cells from tumor cells migrating into the intestinal epithelium or from tumor cells left in the intestinal wall at the site of injection.

Experiments to distinguish between these possibilities are now under investigation. It is also an open question whether the immunity is mediated by cells or antibodies. Cytotoxic antibodies have previously (8) been demonstrated in C3H mice hyperimmunized with C3H-L 1/a cells. However, these antibodies could only be demonstrated after 5–7 immunizing injections. The results of the present study indicate that the antibodies produced in ic immunized mice are without any protective or blocking effect in the tumor neutralization test. Experiments by Süssdorf (9) and Nieuwenhuis (10) indicate that the appendix is essential for the antibody-forming potential in the spleen, and Clancy and Bienenstock (11) stated that lymphocytes with surface IgG and IgM receptors from the Peyers patches especially seed to the spleen. This might explain the pronounced effect of splenocytes and the insignificant effect of lymph node cells observed in our experiments, provided that the tumor-neutralizing effect of sensitized splenocytes depends on the transfer of activated B cells. The temporary production of an ascites tumor which regressed after 7–8 days suggests that protection did not depend on passive immunity exclusively. Experiments aimed at answering this question are in progress.

Summary. Lumenal intracecal inoculation of viable malignant cells produces a protective effect against a secondary ip challenge with the same tumor cell. Cells inoculated in the lumen of the cecum showed immunizing properties as demonstrated by specific antibodies evidenced by a positive indirect immunofluorescence test.

Furthermore, the protective effect can b

ed to an untreated animal by sensi-
nocytes.

rk was supported by the Danish Cancer

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Received July 26, 1976. P.S.E.B.M. 1977, Vol. 154.

The Physiology and Pathology of Human Aging

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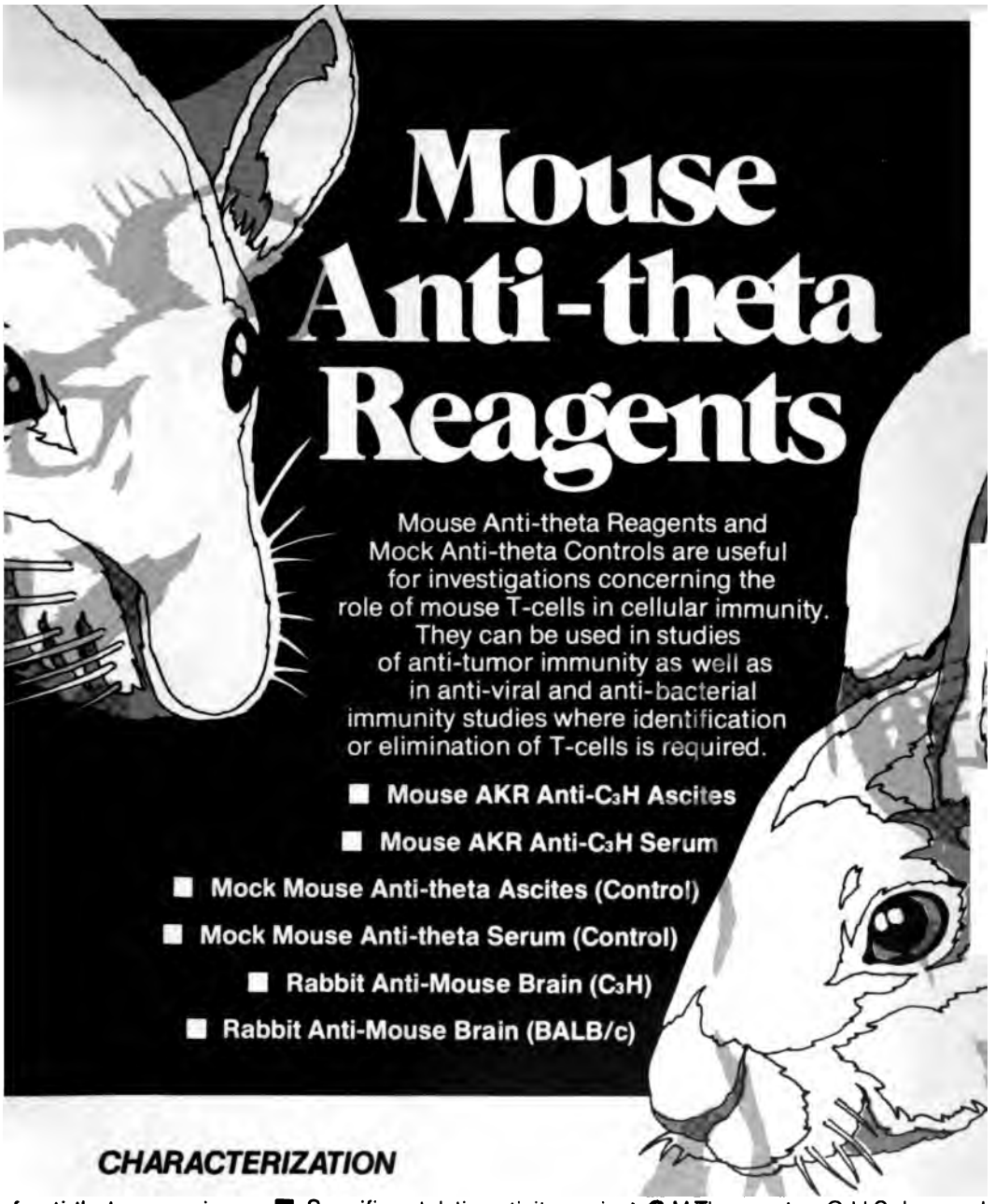
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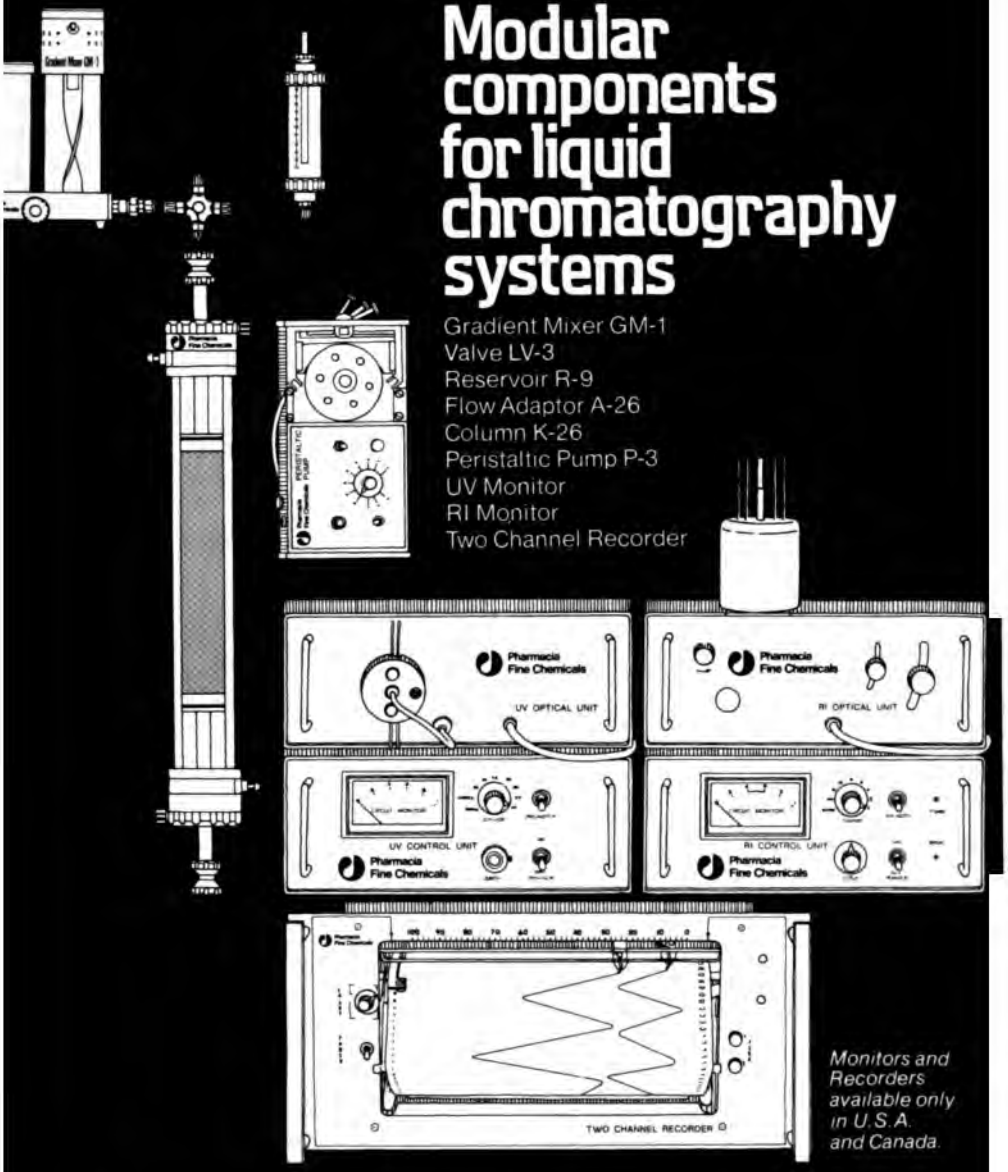

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Volume 154, Number 3, March 1977

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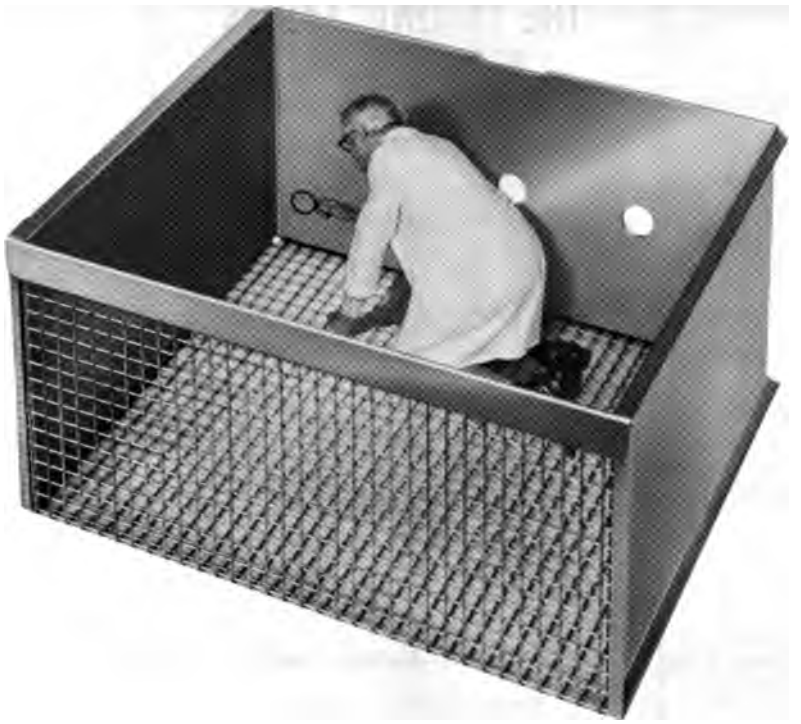

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THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 319-321 (1977)

ɨ-Hydroxy-3-Methylglutaryl CoA Reductase Activity in Germfree Rats (39662)

EINARSSON, JAN-ÅKE GUSTAFSSON, AND BENGT E. GUSTAFSSON

of Medicine, Serafimerlasarettet, Department of Chemistry and Department of Germfree Research, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

s investigations have shown that rats have a reduced fecal excretion ds and neutral steroids and higher serum and liver cholesterol com- h conventional rats (1-4). In ac- with this finding the 7α-hydroxyl- holesterol, which is the rate-limit- ı the conversion of cholesterol to tends to be decreased in germfree The rate-determining step in the sis of cholesterol is the formation ınic acid from HMG CoA (3-hy- ıethylgutaryl CoA) (6). Recently s have been described for deter- : activity of the HMG CoA reduc- In order to further clarify the ns behind the differences in cho- etabolism between germfree and nal rats, it was considered of im- :o investigate the cholesterol syn- e present paper describes the ac- he HMG CoA reductase in liver es from germfree and conven-

ls. [Glutaryl-3-^{14}C]HMG CoA adioactivity, 20 μCi/mg) and DL- c-5-^{3}H(N)]mevalonic acid (diben- ediamine salt) (specific radioac- μCi/mg) were obtained from New Nuclear Corp., Boston, Mass. The ely labeled HMG CoA was di- ı unlabeled material, synthesized Biochemicals, Inc., Milwaukee, eld a specific radioactivity of 1.45 Jnlabeled DL-mevalonic acid lac- DP, glucose 6-phosphate and glu- osphate dehydrogenase were ob- n Sigma Chemical Co., St. Louis,

:. Animals and preparation of mi- Three germfree and three con- male rats of the AGUS[1] strain about 100 days of age and weighing about 250-300 g were used. The germfree animals were reared according to the technique of Gustafsson (8, 9) and fed a standard diet *ad libitum* (9). This diet is semisynthetic with 10% (w/w) arachis oil as source of fat and contains 0.004% (w/w) cholesterol. Conventional animals of the same strain were reared outside the germfree isolators on the same sterilized diet. The germfree animals were killed between 9 and 10 AM by a blow on the head immediately after they had been taken out of the germfree isolators; the conventional animals were killed in the same way at the same time. The livers were removed immediately and chilled on ice. Liver homogenates (10%, w/v) were prepared with a Potter-Elvehjem homogenizer equipped with a loosely fitting Teflon pestle. The homogenizing medium contained: sucrose, 0.3 *M*; nicotinamide, 0.075 *M*; EDTA, 0.002 *M*; and mercaptoethanol, 0.02 *M*. The homogenate was centrifuged at 20,000*g* for 15 min. The microsomal fraction was obtained by centrifuging the 20,000*g* supernatant fluid at 100,000*g* for 60 min. The microsomes were washed with the homogenizing medium and centrifuged again at 100,000*g* for 30 min. The microsomal fraction was then suspended in the homogenizing medium in a volume corresponding to that of the 20,000*g* supernatant fluid. The protein concentration was determined by the method of Lowry *et al.* (10).

Assay of HMG CoA reductase activity. The assay system used was similar to that described by Shefer *et al.* (7). The complete system contained in a volume of 0.85 ml: 0.2 ml of microsomal fraction; phosphate buffer, pH 7.2, 100 m*M*; NADP, 3 m*M*; glucose 6-phosphate, 10 m*M*; glucose 6-phosphate dehydrogenase, 5 enzyme units;

: strain of Long Evans origin has been r germfree conditions at the Department It has later been established at Laboratory Animal Center, Carshalton, England and labeled AGUS.

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[3-^{14}C]HMG CoA, 0.2 m*M*. The incubation was run for 15 min at 37°C. At the end of this period 0.01 μCi of tritium-labeled mevalonic acid was added as an internal standard together with 3 mg of unlabeled mevalonic acid lactone and the reaction was stopped by the addition of 0.1 ml of 5 *M* HCL. The incubation mixture was further shaken for 30 min at 37°C. After cooling to room temperature, 0.5 ml of absolute ethanol was added and the mixture was dried over anhydrous Na_2SO_4. The solution was extracted three times with 3 ml of ethyl ether. The ethyl ether layers were combined, dried over anhydrous Na_2SO_4 and evaporated. The residue, dissolved in acetone, was subjected to thin-layer chromatography (precoated silica-gel plates; 250 μm; Merck, Darmstadt, Germany) with benzene–acetone (1:1, v/v) as solvent. The chromatoplate was sprayed with 0.2% (w/v) solution of 2,7-dichlorofluorescein in methanol and the mevalonic acid lactone zone was located under uv-light. The mevalonic acid lactone band was scraped off into a counting vial. The radioactivity was determined in a Packard Liquid scintillation spectrometer, Model 3003, using Instagel as a scintillator liquid. Corrections were made for quenching by using suitable ^{3}H- and ^{14}C-labeled standards. Corrections were also made for losses during extraction and thin-layer chromatography procedures by the internal standard (tritium-labeled mevalonic acid lactone).

Results. The amount of formed [3-^{14}C]mevalonic acid lactone was expressed as picomoles per milligram of protein per minute. As seen in Table I the labeled mevalonic acid lactone formed in conventional rats amounted to 50.5 ± 2.9 pmole/mg of protein/min. In germfree animals the formation of mevalonic acid lactone was 21.7 ± 3.8 pmole/mg of protein/min which is about 40% of the reaction rate found in conventional rats.

Discussion. It is a well-known fact that germfree rats have higher levels of serum and liver cholesterol compared with conventional rats (3, 4, 11). Further, Kellogg and Wostmann (12) have shown that germfree rats fed with only moderate amounts of cholesterol accumulate 2–3 times as much cholesterol in the liver as conventional rats.

germfree and conventional rats by the HMG CoA (3-hydroxy-3-ıtaryl CoA) reductase activity in nicrosomal fraction. Germfree rats reased activity (21.7 ± 3.8 pmole/ ɔtein/min) compared with conven-ts (50.5 ± 2.9). The results are in relation to present knowledge terol metabolism in the two types s.

k was supported by grants from the Swedish :search Council (16X-206) and from Loo)sterman's Stiftelse.

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Received July 19, 1976. P.S.E.B.M. 1976, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 322-324 (1977)

A Soluble Peroxidase in Heart and Skeletal Muscle of Rat and Mouse[1] (

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The existence of peroxidase in both soluble and insoluble forms was first noted by Willstätter and Pollinger (1) in horseradish, and later by Bancroft and Elliott (2) in animal tissues. The demonstration of true peroxidase activity in many animal tissues is complicated by the concomitant presence of hemoglobin, which has peroxidatic activity, and of catalase, which not only has peroxidatic activity, but also actively degrades the H_2O_2 which is needed for the activity of peroxidase.

The relatively recent advent of techniques for isoelectric focusing on polyacrylamide gel now permits the definitive physical separation of these several proteins. We wish to report here the existence of soluble peroxidatic activity in rat and mouse heart and skeletal muscle, and the absence of comparable soluble peroxidatic activity from a variety of other tissues.

Materials and methods. Mice used were C3H/An1 adults of both sexes; no sex-dependent differences in peroxidatic activity were noted. Rats were adult Charles River Sprague-Dawley males. Mice were killed by cervical dislocation; rats, by ether inhalation. Desired tissues were removed, washed briskly in cold distilled water, blotted on filter paper, homogenized in glass-Teflon Potter-Elvehjem homogenizers with 9 vol of cold 0.25 *M* sucrose, and centrifuged at 48,000*g* for 30 min. The supernatant fractions were used to obtain the results described below; some work with the precipitate fraction is also discussed.

Isoelectric focusing on polyacrylamide gel was performed using the Multiphor apparatus (LKB Produkter, Bromma, Sweden), modified as described elsewhere (3). Peroxidase activity was detected by covering individual gel slabs with a mixture c 0.06% H_2O_2 and unbuffered 0.2 dine hydrochloride. Homovanillic *o*-dianisidine were also tested as and gave comparable results; howe zidine was by far the more sensiti

Chemicals purchased were the h ity available from common sources not further purified.

Results and discussion. Figure 1 of photographs, with counterpart for clarity, demonstrating peroxid ity in the soluble fraction of mou and its absence from the soluble fr mouse liver and lung. A sample hemoglobin (lysed erythrocytes) cluded (first slide), and it can be s the figure that hemoglobin was p each soluble fraction, but other p bands were detected only in heart a liver or lung.

Figure 2 shows that rat heart an muscle contain soluble peroxidase that are not hemoglobin. Two of heart muscle peroxidase isozymes be identical to the two present ir muscle.

Overlapping activities of the heme proteins is a frequent sourc culty and ambiguity in the measur peroxidase activity. Various te have been employed to remedy t tion. For example, the peroxidati of hemoglobin is frequently correct measuring the hemoglobin present culating the peroxidatic activity amount of hemoglobin (4). Also chlorophenol has sometimes been peroxidase assay mixtures to inhibi activity (5).

The clearest demonstration of p activity, however, would seem t physical separation of the various which is readily accomplished by is focusing on polyacrylamide gel. H bin has an isoelectric point (p*I*) s over the range of about pH 6.9-7.

[1] Work supported by the United States Energy Research and Development Administration, and by the Ministerio Educacion y Ciencia, Spain.

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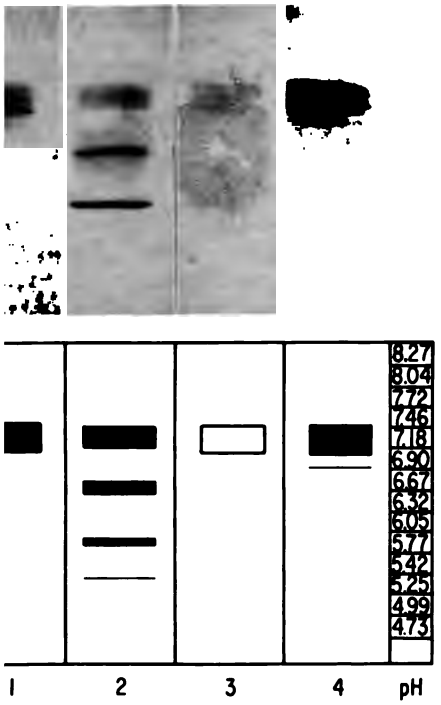

1. Isoelectric focusing of mouse hemoglobin erythrocytes) and soluble fractions from heart, ınd lung. Drawings below are counterparts of raphs above, for easier visualization. Slide 1: erythrocytes (diluted by visual inspection to the imate color of the heart extract). Slide 2: Solu-ction of heart. Slide 3: Soluble fraction of liver. : Soluble fraction of lung. For each sample, 25 applied to the gel. The cathode is at the top; at the bottom.

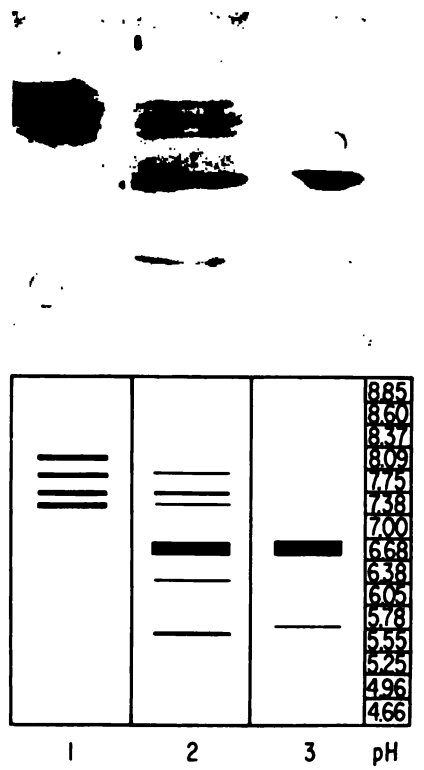

FIG. 2. Isolectric focusing of rat hemoglobin (lysed erythrocytes) and soluble fractions of heart and skeletal muscle. Slide 1: Lysed erythrocytes. Slide 2: Soluble fraction of heart. Slide 3: Soluble fraction of skeletal muscle. Details are as in Fig. 1, except that soluble fractions from 10% homogenates were used in Fig. 1, and from 20% homogenates in Fig. 2.

:, and about pH 7.4–8.1 in the rat. ıse, which is readily identified on the graphs by the area of oxygen bubbles, somewhat lower p*I*. Catalase is well ı for its diffuse banding, either isoe-ally or on standard polyacrylamide or electrophoresis gels; this diffuseness lily to be seen in a multitude of recent s (for examples, see 6, 7). The most l lines (pH 6.7, 5.8, and 5.3) in Fig. 1 ɔe considered to represent true peroxi-

nmercial beef heart cytochrome *c*, at ıg/ml, produced no lines on the gels the conditions employed. This is not ıexpected finding, because the p*I* of ırome *c* has been stated (8) to be 9.8 ;her; this is beyond the range of the sed. The possibility that the benzidine ng is due to some *nonperoxidase* hem-oprotein other than hemoglobin, catalase, or cytochrome *c* cannot be ruled out at present. We have not, however, been able to specify any other known hemoprotein that might be responsible.

Bancroft and Elliott (2), in measuring the peroxidase activity of a variety of tissues from the rat and the rabbit, used well-pulverized whole tissue. Neufeld *et al.* (9) used only the resuspended insoluble portion of tissues for peroxidase assays. Bancroft and Elliott (2) found lung and spleen to have the greatest peroxidase activity of any tissues tested. Neufeld *et al.* (9) found lung and spleen to have 20–30 times the peroxidatic activity of heart and skeletal muscle; Bancroft and Elliott (2) did not test these latter two tissues. After isoelectric separation on polyacrylamide gels, we found in mouse or rat lung, spleen, liver, kidney, or brain no

soluble peroxidatic activity that could not be accounted for as hemoglobin. We also attempted to solubilize the insoluble peroxidase of lung and spleen with urea or chlorhexidine, as we successfully did with dog and pig thyroid peroxidase; however, no true peroxidase lines could be demonstrated on Multiphor gels.

We suggest that the discrepancy between the present results and those of others lies in the fact that intact erythrocytes remain in insoluble fractions of tissues prepared with isotonic or hypertonic extractive agents. In our experience, the insoluble fractions of lung and spleen, prepared with 0.25 *M* sucrose, showed strong coloration and strong peroxidatic activity with benzidine as substrate when resuspended in distilled water; however, when the resuspended material was centrifuged, the new supernatant continued to exhibit color, but on Multiphor gels the only "peroxidase" bands were those cathodic lines (pH 6.9–7.4) that we associate with hemoglobin.

In mouse heart, on the other hand, soluble peroxidase activity was found by the benzidine assay. We measured the hemoglobin content of the preparation and corrected for the peroxidatic activity of the hemoglobin. It was found that the hemoglobin could account for *all* peroxidase activity, yet on Multiphor definite nonhemoglobin peroxidase lines were observed. This suggests that the commonly used correction for the peroxidatic activity of hemoglobin is at semiquantitative measure.

Summary. Several peroxidase isc have been demonstrated in the 0.25 crose-soluble portion of mouse a heart and skeletal muscle. Isoelectric ing on polyacrylamide gel was used t that the peroxidatic activity is not hemoglobin, catalase, or cytochrome true peroxidase activity was detec either the soluble or insoluble fract mouse liver, kidney, brain, spleen, o

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Received September 1, 1976. P.S.E.B.M. 197 154.

ICS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 325-330 (1977)

ts of Bile Acids, Lecithin, and Monoolein on Amino Acid Absorption from the Human Duodenum[1](39664)

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: acids inhibit water absorption and olyte transport from the colon of hu- (1) and canines (2) and from the je- ı of hamsters (3); and they inhibit glu- ıbsorption from the human jejunum). Although taurochenodeoxycholic 2.5 m*M*) has been said to inhibit ab- on of L-leucine and L-lysine from the n jejunum (6), and the unconjugated :id deoxycholate inhibits absorption of and leucine from the rat intestine (7, e effects of other bile acids on absorp- f essential amino acids (EAAs) have een studied. The addition of lecithin hes the effects of bile acids upon water nent and electrolyte transport in the n jejunum, but the addition of oleate ıot restore normal glucose absorption. :ffect of lecithin and monoolein on absorption has not been reported.

:e intraduodenal concentrations of ; are not equal, we elected to study sorption of unequal molar mixtures of ;, similar to the composition of casein ; (9) protein hydrolysate. The absorp- ate of such EAA mixtures from the ı and transplanted human duodenum nd from the human jejunum (11) has reported previously. The jejunal ab- ɔn rates of individual amino acids in mixtures are very similar to the rates uimolar EAA mixtures (12). Again, fects of bile acids, lecithin, or mono- on absorption of individual amino in these mixtures of EAAs have not eported.

aims of our study were: (1) to estab- ıe duodenal absorption kinetics of ; (2) to determine the effects of tau- onjugated bile acids (cholic, cheno- :holic, and deoxycholic) on duodenal EAA absorption; (3) to determine whether monoolein alters duodenal EAA absorption; and (4) to determine whether the addition of lecithin or monoolein to the perfusion mixture of bile acid and EAAs may affect duodenal EAA absorption.

Materials and methods. Fifty healthy male volunteers participated in these studies. After an overnight fast, a double-lumen polyethylene tube was placed intraduodenally under fluoroscopic observation. Isotonic saline or test solutions containing a non-absorbable marker – polyethylene glycol (PEG), 5 g/liter – were perfused into the second part of the duodenum at a constant rate (10 ml/min). During steady-state conditions, specimens of duodenal contents were siphoned from near the ligament of Treitz, 20 cm distal to the infusion site. Perfusates were collected over ice and pooled at 20-min intervals. In each study, isotonic saline was perfused 60 min initially for cleansing; and then either a control (isotonic saline) or test solution was perfused for 100 min. The test perfusates contained EAAs alone; EAAs in combination with sodium taurocholate (TC), sodium chenodeoxycholate (TCDC), or sodium taurodeoxycholate (TDC); and EAAs in combination with TC + monoolein (MO) and with TC + lecithin. The 78 m*M* EAA perfusate contained (in m*M*) 10.65 threonine (Thr), 12.95 valine (Val), 5.15 methionine (Met), 11.25 isoleucine (Ileu), 16.8 leucine (Leu), 6.7 phenylalanine (Phe), 13.45 lysine, and 1.05 tryptophan. Tryptophan was not measured by our techniques and not included in our study. In all studies, gastric juice was aspirated continuously from the antrum by an additional tube.

The volumes of gastric and duodenal aspirates and their pH and PEG concentrations (13) were measured. Duodenal EAA concentrations were determined by methods previously described (10) and EAA absorptions were calculated from their concentra-

ported in part by Research Grant AM-6908 e National Institutes of Health, Public Health

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1977 by the Society for Experimental Biology and Medicine erved.

ISSN 0037-9

tions relative to PEG in duodenal samples. Only aspirates collected after the first 40 min of test perfusion were utilized for these determinations.

Since the rate of perfusion and the composition of the perfusate in each trial were known constants, the rates of administration of EAAs could be expressed as μM/min; and the calculated amounts recovered during collection intervals of known length could be expressed in the same terms. The difference between the rates of perfusion and recovery represented the rate of absorption by the duodenum. In addition, we have expressed our results of percent absorption of individual amino acid and the total EAA by the formula:

$$\text{Percent absorption} = \frac{\text{Rate of EAA absorbed in } \mu M/\text{min}}{\text{Rate of EAA perfused in } \mu M/\text{min}} \times 100.$$

For determining intrastudy variability in the absorption of each EAA, the last three 20-min collections from each of five subjects were compared; and the average coefficient of variance was between 7 and 15%.

EAAs were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; sodium TC, sodium TCDC, and sodium TDC were synthesized from taurine (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) and cholic acid, chenodeoxycholic acid, and deoxycholic acid, respectively (Matheson Coleman & Bell, East Rutherford, N.J.) by methods of Hofmann (14). The products migrated as single spots on thin-layer chromatography. Emulsified monoolein consisted of 10 m*M* of 1-MO (Eastman Organic Chemicals Div.) in dilute 1 m*M* sodium TC. This solution was sonicated for 60 min with a Biosonik (Will Scientific, Inc., Rochester, N.Y.) to emulsify the MO into solution. Thin-layer chromatography showed that the preparation was more than 95% monoglyceride, and recoveries from the perfusates showed that more than 90% of the monoglyceride was hydrolyzed to fatty acid during the study. Lecithin (chromatographically pure, Schwartz/Mann Division, Becton, Dickinson and Co., Orangeburg, N.Y.) *was used as purchased.* When EAA, MO, lecithin, or bile acids were combined in test solutions, the original molar concentrations were preserved. All perfusates were prepared immediately before use, made isotonic with sodium chloride, adjusted to pH 6.3, and warmed to 37°C.

The validity and reproducibility of the perfusion technique and coefficients of variations of PEG determinations have been detailed elsewhere (13, 15).

Experimental design. 1. To determine EAA duodenal absorption, five subjects each were given four 100-min perfusions which (in random order) contained 13, 26, 52, and 78 m*M* EAA mixture. 2. To determine intrastudy variation, three collections made during the 78 m*M* EAA perfusion of each of the five subjects were analyzed. 3. To test the effect of bile acid upon absorption of 78 m*M* EAA from the duodenum, 10 m*M* TC, TCDC, and TDC were perfused with 78 m*M* EAA in 7, 3, and 3 subjects, respectively; and the collections were compared with those from 11 subjects who had received 78 m*M* EAA alone. In addition, the effect of 5 m*M* TC was tested in five studies. 4. To determine the effect of fat upon duodenal absorption of 78 m*M* EAA, 10 m*M* monoolein emulsified with 1 m*M* TC was perfused with 78 m*M* EAA in six subjects. 5. To test whether the effect of 10 m*M* TC upon EAA absorption could be abolished, 10 m*M* MO was added to the mixture of 10 m*M* TC and 78 m*M* EAA in five cases and 2.5 m*M* lecithin was added to the same mixture in five other cases. 6. Lastly, to determine how influx of EAA into the duodenum is influenced by pancreozymatic and cholecystokinetic effects of intraluminal fat and EAA (13, 16), EAA output into the duodenum was measured during normal isotonic saline perfusion and during perfusion with normal isotonic saline and cholecystokinin-pancreozymin (CCK) intravenous stimulation in three subjects.

Results. Absorption of EAA mixture from duodenum. Absorption of the total EAA mixture and of individual amino acids in the mixture is given as percentages in Table I. The duodenum absorbed 50% of the total mixture. Individually, the absorption of valine, isoleucine, leucine, and phenylalanine approximated this rate; that of methionine was slightly more; and that of lysine and

TABLE I. ABSORPTION RATES OF EACH PERFUSED EAA AND TOTAL MIXTURE: IN μM/MIN (AND PERCENTAGE), MEANS ± SE.

Perfusate (all units mM)	N	Thr	Val	Met	Ileu	Leu	Phe	Lys	Total
					Non-paired studies				
Endogenous EAA,[a] with									
Isotonic saline	3	−1.5 ± 0.3[b]	−2.1 ± 0.6[b]	−0.7 ± 0.2[b]	−1.7 ± 0.5[b]	−2.6 ± 0.7[b]	−1.3 ± 0.3[b]	−1.9 ± 0.5[b]	−11.7 ± 3.2[b]
		(−4 ± 1)	(−3 ± 1)	(−2 ± 1)	(−3 ± 1)	(−3 ± 1)	(−4 ± 1)	(−3 ± 1)	(−3 ± 1)
Isotonic saline + iv CCK	3	−8 ± 3[b]	−11 ± 3[b]	−7 ± 4[b]	−7 ± 2[b]	−10 ± 5[b]	−7 ± 2[b]	−14 ± 4[b]	−70 ± 15[b]
		(−21 ± 8)	(−16 ± 5)	(−7 ± 2)	(−11 ± 3)	(−11 ± 5)	(−24 ± 7)	(−23 ± 6)	(−18 ± 4)
Control:	11	38 ± 4	67 ± 5	31 ± 2	62 ± 4	93 ± 7	31 ± 2	58 ± 4	381 ± 27
78 EAA		(36 ± 4)	(53 ± 4)	(60 ± 4)	(56 ± 4)	(55 ± 4)	(46 ± 4)	(44 ± 3)	(50 ± 4)
Tests:									
78 EAA, with									
+ 1 TC	6	33 ± 9	66 ± 13	30 ± 5	65 ± 8	87 ± 18	28 ± 6	41 ± 16	351 ± 67
10 MO		(31 ± 8)	(51 ± 10)	(57 ± 10)	(57 ± 7)	(52 ± 11)	(43 ± 9)	(41 ± 12)	(46 ± 9)
+ 10 TC	5	36 ± 11	59 ± 14	26 ± 7	51 ± 14	76 ± 21	30 ± 5	50 ± 15	345 ± 89
10 MO		(34 ± 10)	(46 ± 11)	(50 ± 13)	(45 ± 13)	(45 ± 13)	(45 ± 8)	(51 ± 12)	(48 ± 12)
+ 10 TC	5	32 ± 6	52 ± 9	26 ± 3	50 ± 8	79 ± 10	25 ± 4	61 ± 12	325 ± 44
2.5 lecithin		(30 ± 5)	(40 ± 7)	(51 ± 5)	(45 ± 7)	(47 ± 6)	(37 ± 6)	(46 ± 9)	(42 ± 6)
+ 10 TCDC	3	31 ± 9	46 ± 13	24 ± 5	53 ± 17	67 ± 16	20 ± 6	41 ± 13	282 ± 79
		(29 ± 8)	(36 ± 10)	(47 ± 10)	(47 ± 15)	(40 ± 10)	(30 ± 10)	(31 ± 10)	(37 ± 10)
+ 10 TC	7	13 ± 3[b]	36 ± 5[b]	16 ± 2[b]	34 ± 4[b]	48 ± 6[b]	15 ± 3[b]	33 ± 6[b]	193 ± 27[b]
		(12 ± 3)	(28 ± 4)	(31 ± 4)	(30 ± 4)	(28 ± 4)	(21 ± 5)	(24 ± 4)	(25 ± 4)
+ 5 TC	5	16 ± 5[b]	23 ± 7[b]	12 ± 6[b]	27 ± 6[b]	42 ± 10[b]	13 ± 4[b]	26 ± 9[b]	161 ± 9[b]
		(15 ± 5)	(29 ± 6)	(23 ± 12)	(24 ± 5)	(25 ± 6)	(19 ± 5)	(19 ± 6)	(21 ± 5)
+ 10 TCDC	3	13 ± 5[b]	20 ± 7[b]	14 ± 3[b]	20 ± 6[b]	51 ± 15[b]	9 ± 2[b]	15 ± 8[b]	141 ± 31[b]
		(12 ± 4)	(15 ± 5)	(27 ± 6)	(18 ± 6)	(30 ± 9)	(13 ± 3)	(11 ± 7)	(18 ± 4)
					Paired studies				
EAA: 13	5	5 ± 1[b]	8 ± 2[b]	4 ± 1[b]	7 ± 2[b]	11 ± 3[b]	3 ± 1[b]	6 ± 2[b]	46 ± 12[b]
26	5	10 ± 2[b]	18 ± 4[b]	8 ± 2[b]	16 ± 4[b]	24 ± 6[b]	8 ± 2[b]	15 ± 4[b]	100 ± 22[b]
52	5	22 ± 2[b]	42 ± 3[b]	19 ± 1[b]	39 ± 2[b]	57 ± 3[b]	18 ± 1[b]	30 ± 3[b]	229 ± 14[b]
78	5	34 ± 4	61 ± 4	30 ± 3	58 ± 4	83 ± 7	28 ± 2	45 ± 9	346 ± 25

[a] Minus sign indicates endogenous EAA secreted under saline perfusion, without and with iv CCK stimulation.
[b] Significantly different ($P < 0.01$) from control (78 mM EAA), by rank sum methods.

threonine was somewhat less.

In kinetic studies (Table I) of the five subjects who received mixtures totaling 13, 26, 52, and 78 m*M* EAA, a direct linear relationship was found between the total perfused and the total absorbed. Of individual EAAs (Fig. 1), it was observed that methionine was absorbed at the most rapid rate, while the branch-chained amino acids —isoleucine, leucine, and valine—were absorbed slightly less rapidly, and lysine and threonine at the slowest rates.

Effect of taurine-conjugated bile acids on EAA (Table I). TC and TDC significantly depressed EAA absorption ($P < 0.01$). Both 5 and 10 m*M* concentrations of TC depressed total EAA absorption by 50%, while TDC decreased it 65%. TCDC depressed EAA absorption by only 26%, which was not statistically significant. The inhibition of EAA absorption by TC and TDC affected all individual amino acids similarly.

Effect of MO and lecithin on EAA absorption (Table I). Emulsified MO alone had no significant effect on EAA absorption. The addition of MO to the perfusion mixture of EAA and 10 m*M* TC restored EAA absorption to normal. Similarly, the addition of 2.5 m*M* lecithin to the mixture of 78 m*M* EAA and 10 m*M* TC restored EAA absorption to normal.

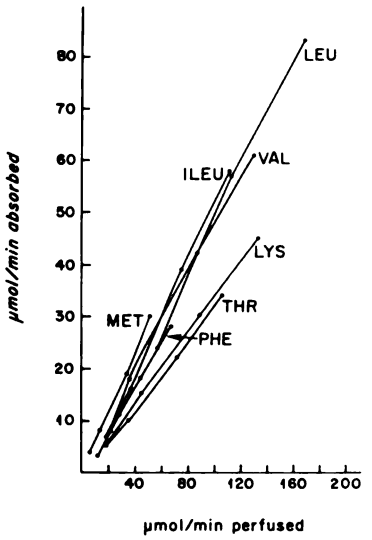

FIG. 1. Duodenal absorption of component EAAs from mixture perfused (see Table I for composition): *Means from five subjects.*

ut BA. It is known that EAA perfu-
f the duodenum stimulates biliary and
eatic secretion. When we stimulated
ancreas with exogenous CCK, EAAs
ecreted endogenously. Therefore, it is
that our percent absorption of EAA is
% lower than the true value and that
ercent absorptions in the human duo-
n and jejunum are very similar for this
ular EAA mixture.
ontrast, taurine-conjugated bile acids
ed with the EAAs inhibit pancreatic
iliary secretion (16); and the reported
ption rates of the bile acid-EAA com-
ons are believed to be accurate and
ely unaffected by endogenous EAA.
ur studies conjugated trihydroxy (TC)
lihydroxy (TDC) bile acids inhibited
absorption. This observation with
is in agreement with results of *in vivo*
) and *in vitro* (7) animal experiments.
ver, inhibition of amino acid absorp-
as not been observed with TC bile
(7, 19). Although contamination of
lt preparations with deoxycholate has
incriminated in the past as a cause for
tion of water-soluble nutrients (7),
reparations of TC were at least 99%
by thin-layer chromatography. Fur-
ore, identical preparations of TC in
studies from this laboratory (20, 21)
to exhibit any effect on net water
ment. Therefore, it is extremely un-
that TDC contamination of TC caused
minution of EAA absorption when we
ed EAA with TC.
w bile acids inhibit EAA absorption
ow lecithin and MO abolish this inhibi-
re not known and have not been eluci-
by our studies. Bulk water flow, ef-
on adenosine triphosphatase (ATP-
and disruption of cell membranes may
olved.
nvection (solvent drag, bulk water
is probably important in EAA absorp-
It has been demonstrated that convec-
n the direction of absorption is impor-
n carbohydrate absorption; but the
ction component of carbohydrate ab-
on during osmotic secretion is very
(22). Glucose absorption is depressed
DC, which induces net water move-
(5); but it is not depressed significantly
water movement is enhanced with
mannitol (5). Furthermore, TC – which in-
hibits EAA absorption – does not have any
effect on net water movement (20). There-
fore it is unlikely that water movement in-
duced by bile acids is the only or major
factor that inhibits EAA absorption.

Bile acids may influence chemical mecha-
nisms proposed for active transport of elec-
trolytes (4). Recently it has been shown that
glycine- and taurine-conjugated bile salts
inhibit Na^+,K^+-stimulated rat intestinal mu-
cosal ATPase (23) and stimulate adenylate
cyclase activity of human colonic mucosa *in
vivo* (24). Whether these properties of bile
acids cause inhibition of EAA absorption is
unknown. However, the effects of individ-
ual bile acids upon ATPase activity do not
correlate well with their effects upon water
and electrolyte absorption (4, 25). There-
fore, the assumption that inhibition of active
transport by bile acids is related to their
effects on ATPase is not well substantiated.

It is well known that bile acids interrupt
cell membranes (25). Altered surface mor-
phology of intestinal mucosa exposed to de-
oxycholate has been demonstrated (2, 3);
and these changes may be related to reduc-
tion of EAA absorption observed with the
EAA + TDC perfusion. Lecithin or MO
may abolish the bile salt inhibition of EAA
absorption by causing less bile salt to be
available at the surface of the mucosal cells.
Recently, it has been demonstrated by an
equilibrium-dialysis method that intermicel-
lar bile salt concentration decreases as phos-
pholipid content of mixed micelles increases
(26). Therefore, it is postulated that when
either MO or phospholipid (lecithin) is in
the perfusing solutions, micelles are en-
larged – and consequently incorporate more
bile acids within them and allow less bile salt
to act at the surface of mucosal cells. Dis-
ruption of cell membranes is thereby pre-
vented, and EAA absorption proceeds nor-
mally.

Summary. The effects of bile acid, leci-
thin, and monoolein (MO) on essential
amino acid (EAA) absorption from the hu-
man duodenum were determined. It was
found that: (1) methionine was absorbed at
the most rapid rate while isoleucine, leu-
cine, and valine were absorbed slightly less
rapidly and lysine and threonine at the slow-
est rates; (2) duodenal EAA absorption

rates are very similar to previously reported rates for jejunal absorption of EAA; (3) taurocholate (TC) and taurodeoxycholate significantly depressed EAA absorption; and (4) although emulsified MO had no significant effect on EAA absorption, the addition of MO or lecithin to the EAA + 10 m*M* TC perfusion mixture restored EAA absorption to normal. How bile acids inhibit EAA absorption and how lecithin and MO abolish this inhibition are not known and have not been elucidated by our studies. However, it is postulated that when either lecithin or MO is present, less bile salt is available to act at the surface membranes of intestinal cells, disruption of cell membranes is prevented, and EAA absorption proceeds normally.

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Received September 1, 1976. P.S.E.B.M. 1
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ΠΕ SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 331-336 (1977)

ɔn the Growth Limiting Mechanism in the Rat Ventral Prostate (39665)

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e accessory sex glands are under ontrol of androgens, and the vente in rats is no exception. Thus ic lobe is involuted after orchiecffect which depends on the activaɔphagic processes in the epithelial ting in autolysis and loss of funcormance (1, 2). Exogenous ansuch rats will initiate DNA syncell proliferation. However, when r of cells is restored to normal, ıesis is curtailed and cell proliferedly reduced (3, 4). Hence, the e growth of the prostate (but not y ability) becomes refractory to sive androgen stimulation. This ıe existence of a probable homeoraint mechanism in the prostate. t possible inhibitory mechanisms tly been discussed by Bruchovsky vho considered the production of 6) from the prostatic epithelial attractive possibility to explain ive feedback on proliferative the prostatic epithelial cells prood-borne tissue-specific growth a chalone) in a precise amount mal number of cells sufficient to ɔroliferation, then proliferation ır only when the number of cells ɔelow the normal level and would ted in the regenerating prostate ormal number of cells had been

for the hypothetical existence of chalone has been furnished by ıical studies on the effects of a xtract on patients with benign yperplasia. Treatment with such , possibly containing a growthprostatic chalone, was shown to ficant symptomatic improvement lerable percentage of patients in stages of the disease but not in ages (7–10). Conclusive experimental results demonstrating the existence of a prostatic chalone are, however, still lacking, and the purpose of this study was to provide experimental evidence for the existence or nonexistence of a prostatic chalone regulating prostatic growth in rats.

Materials and methods. Adult male Wistar–Furth rats, 250–350 g, obtained from Microbiological Laboratories, Bethesda, Maryland, were used in the experiments. The animals, which are closely inbred, were kept under standard conditions and had free access to food and water.

In the first experiment 20 rats were divided at random into four groups. Half of the ventral prostate in the rats in two of the groups was extirpated under ether anesthesia. The extirpated part of the ventral prostate in each rat was weighed and its DNA content determined (11). The rats in the two other groups underwent sham operation. Two weeks and four weeks after the operation, the animals in one group with hemiprostatectomized rats and the animals in one group with sham operated rats were sacrificed. The ventral prostate and other accessory sexual glands were dissected and weighed. The DNA content in the ventral prostate was determined.

In the second experiment 30 rats were divided at random into six groups. The rats in group 1 underwent sham operation, those in groups 2, 3, and 5 were orchiectomized and sham operated, and those in groups 4 and 6 were orchiectomized and had half of their ventral prostate extirpated. The extirpated part of the ventral prostate of each of the rats in groups 4 and 6 was weighed and its DNA content determined. Two weeks after the operations the rats in group 2 were sacrificed and the ventral prostate and other accessory sexual glands were dissected and weighed. The DNA content in the ventral prostate was determined. The rats in groups 5 and 6 were injected intramuscularly with

by the Society for Experimental Biology and Medicine

ISSN 0037-97

100 mg/kg of testosterone hexyloxyphenylpropionate (Andradurin, AB Leo, Helsingborg, Sweden), whereas the rats in groups 1, 3, and 4 received an intramuscular injection (2 ml/kg) of the solvent, isopropyl myristate (ICN Pharmaceuticals Inc., Plainview, New York). Two weeks after the injection, all rats were sacrificed and the ventral prostate and other accessory sexual glands were dissected and weighed. The DNA content in the ventral prostate was determined.

In the third experiment 30 rats were orchiectomized and divided at random into three groups. Two weeks after the operation, the rats in the first group were sacrificed. The atrophied ventral prostate was dissected, divided into its two lobes, and implanted into the spleen and between the muscles of the neck (one lobe at each location) of the rats in the second group. Thus, as a result of the implants each animal had more prostate cells than are normally found in orchiectomized animals. Small pieces of the liver were implanted into the spleen and between the muscles of the rats in the third group. The rats were injected intramuscularly with 100 mg/kg of testosterone hexyloxyphenylpropionate. Two weeks later the rats were sacrificed and the ventral prostate and other accessory sexual glands were dissected and weighed. Also the prostatic implants were dissected and weighed. The DNA content of the dissected prostatic tissue was determined. The prostatic implants in one rat of the ten in the second group were taken for histological examination.

Results. The results from the first experiment showed that even after as long a time as 28 days, the ventral prostate in hemiprostatectomized rats had not regenerated back to the weight of that in the control rats. The mean weight in the hemiprostatectomized animals was 170 mg with a standard error of ±13 mg, whereas in the control rats the weight was 289 ± 19 mg. Also the DNA content in the prostate was much lower in the hemiprostatectomized rats, 509 ± 28 μg, than in the control rats, 851 ± 63 μg. When the weight and DNA content of the extirpated part of the ventral prostate in the hemiprostatectomized animals was added to the weight and DNA content of the ventral prostate at sacrifice, the combined weight and the combined content did no significantly ($P > 0.05$) from the wei DNA content of the ventral prostat control rats. This indicates that in regeneration at all had occurred in t tral prostate, even if the number of p epithelial cells have been reduced proximately 50% by the hemipr tomy. Similar results were obtaine the rats sacrificed 14 days after the tion.

In the second experiment orch was shown to cause a profound atr the ventral prostate. The weight duced to 8 and 13% in the hemipr tomized and the sham-operated rats, tively, and the DNA content to 20%, respectively, in comparison v weight and DNA content of the prostate in the control rats.

Androgen treatment of orchiect sham-operated rats initiated 14 da operation reversed the prostatic a The weight and DNA content of the prostate, 321 ± 16 mg and 880 ± respectively, did not differ significan the corresponding weight and conte sham-operated control animals, 27 mg and 847 ± 21 μg. In orchiect hemiprostatectomized rats, howev androgen treatment brought the prostate weight and DNA up to o the normal level, 166 ± 9 mg and 4 μg, a level that could be expecte found in hemiprostatectomized bu wise intact rats. When the weight ar content of the extirpated part of the prostate was added to the weight ar content of the ventral prostate in chiectomized, hemiprostatectomiz drogen-treated rats, the combined and DNA content, 303 ± 15 mg an 29 μg, did not differ significantly f weight and DNA content in the orch ized, sham-operated, androgen-trea 321 ± 16 mg and 880 ± 27 μg. Th tend to indicate that an androgen-restoration of the atrophied ventral could be terminated long before the of prostatic cells had been brought normal.

In the third experiment the nu prostatic cells was artificially incre intrasplenic and intermuscular impl

: ventral prostatic tissue. Andro-
ation caused the implants to grow
ly. At sacrifice, the implants in
pleen and the muscles were well
(Fig. 1). The microscopically ex-
plants had a normal histological
: with tall columnar epithelial
a clear Golgi area and with the
ina filled with secretion. The im-
e well vascularized, and any hor-
etion from the prostatic epithelial
be expected to reach the general
.

ologically observable stimulation
static implants was accompanied
ed increase in weight and DNA
able I). The combined weight of
nts was approximately half the
he rat's own ventral prostate, and
content was approximately two
: weight and DNA content of the
ventral prostate was found to be
in the animals implanted with
ssue as in the animals implanted
ontrol tissue (liver). This means
ts with the prostatic implants had
gher total content of prostatic tis-
ne control rats in which the pros-
had grown to a weight and DNA
at could be expected to be found
rats.

ee experiments the weights of the
al prostate, coagulating glands,
minal vesicles were found to be
by any surgically induced reduc-
rease of the number of cells in the
ostate.

on. The understanding of the reg-
growth and development of tis-
rgans presents a great challenge
ologist. Obviously, a regulatory
n is necessary for organ homeosta-
nust play an important role in the
o injury and some unilateral or-
ations such as nephrectomy. The
ic constraint mechanism is evi-
ective in malignant tissue. The
the regulatory mechanism(s) has
letermined. One attractive theory
ne mitotic activity is controlled by
orne tissue mitotic inhibitor, a
n such a way that the greater the
s within the body space, the lower
rate of cell production (6). This theory has recently been considered an attractive possibility to explain experimental results concerning the proliferation of prostatic epithelial cells (5). In the present study our aim was to obtain evidence for the existence of a prostatic chalone regulating the growth of that organ.

In the first of the three experiments the number of ventral prostatic cells was halved by hemiprostatectomy. If a chalone was secreted from the ventral prostate, the blood level of that mitotic inhibitor would be reduced. As a consequence, the remaining part of the ventral prostate could be expected to grow much in the same way as the liver would do after partial hepatectomy or the remaining kidney would do after unilateral nephrectomy (6, 12). The results clearly show, however, that no growth of the remaining part of the prostate had occurred 4 weeks after operation. This contrasts markedly to the regenerative ability of the rat liver where the normal size was regained within three weeks after removal of more than half of the tissue (13).

In the second experiment hemiprostatectomized, orchiectomized rats were given androgen to induce cell divisions and growth in the atrophied ventral prostate. If the prostate cells secreted a chalone regulating prostatic growth, cell division in the prostate of the hemiprostatectomized rats would not end until the normal cell number was restored. However, at the time of sacrifice, cell division had not progressed beyond the stage when the size and cell number was half that in the control rats and in normal rats.

In the third experiment the number of prostatic cells was increased above normal through implantation of ventral prostatic tissue from other rats. As we used closely inbred rats in all the experiments, there were no immunological difficulties, but the implants grew well after androgen treatment of the castrated recipient rats. If the prostatic cells secreted a chalone regulating prostatic growth, cell division in the ventral prostate of the implanted rats would be expected to end when the total number of prostatic cells in the body was back to normal. However, cell division in the ventral prostate of the implanted rats did not stop at that level but proceeded until the total number of prostatic cells (as reflected by the

A

FIG. 1. Prostatic implant in the spleen 14 days after implantation into orchiectomized rats which then were given androgen. A. The implant which occupies the center part of the spleen has well developed secretion-filled acini with little surrounding fibromuscular stroma (×35). B. In most of the acini the epithelial cells are columnar and well developed with a clear Golgi area and a rounded nucleus with nucleoli (×280).

TABLE I. WEIGHT AND DNA CONTENT OF THE VENTRAL PROSTATE AND OF IMPLANTS OF VENTRAL PROSTATIC TISSUE INTO ORCHIECTOMIZED ANDROGEN-TREATED RATS.[a]

	Ventral prostate		Implants	
Group	Weight, mg	DNA content, μg	Weight, mg	DNA content, μg
A	338 ± 14	880 ± 22	165 ± 16	590 ± 62
B	316 ± 9	862 ± 17	–	–

[a] Orchiectomized rats received implants into the spleen and intermuscularly of the atrophied ventral prostate of other orchiectomized rats (Group A; $n = 9$) or of control tissue (liver) (Group B; $n = 10$). The animals were given a single injection of a long acting androgen, testosterone hexyloxyphenyl-propionate, and were sacrificed 14 days later. The mean values and their SE are given.

DNA content of the prostatic tissue) was 170% of the number in the control rats. At that level, the cell number in the ventral prostate of the implanted rats was the same as the cell number in the ventral prostate of the control rats. Thus, the results of these three experiments show that a prostatic chalone is not the only factor that can inhibit cell divisions in the prostate. In fact, a prostatic chalone seems to have at the most a very subordinate role in the mechanism regulating prostatic growth at least in the rat.

It is evident that further possible regulatory mechanisms must be considered and examined to find the factor that terminates the cell divisions in the prostate. This factor is evidently defective in the malignant prostate. It would appear that an increased knowledge of the regulatory mechanism may give new leads for alternative modes of treatment of prostatic cancer.

Summary. In search for the mechanism that limits the growth of the prostate, experiments were performed to find evidence for the existence of a prostatic chalone. Inbred rats were hemiprostatectomized to reduce the level of a hypothetical circulating chalone. No evidence of regeneration was found 4 weeks after operation. Other hemiprostatectomized, orchiectomized rats were given androgen to induce prostatic growth. Cell division in the ventral prostate ended when the size of the prostate was only half of that in the control rats. Orchiectomized rats with intrasplenic and intermuscular implants of ventral prostatic tissue from other orchiectomized rats were given androgen. The cell divisions in the prostatic tissue did not end until the total cell number was 170% of that in the control rats. The results indicate that other factors than a prostatic chalone have the dominating role in the mechanism that limits prostatic growth.

We wish to express our appreciation to Dr. A. Szolnoky for his precise determination of the prostate DNA content, and to Mr. J. Brachmann for his excellent technical assistance. This work was supported in part by United States Public Health Service Grant No. RR-05648-9 of the National Institutes of Health.

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Received July 12, 1976. P.S.E.B.M. 1976, vol. 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 337-340 (1977)

es in Plasma Glucose and Insulin Levels Induced by Adrenergic Agents in Normal and Alloxan-Diabetic Rats (39666)

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mited ability of isoproterenol to ele-
od glucose levels in normal rats is
ablished (1-3). Previous experi-
om this laboratory have indicated
effects of isoproterenol on the pan-
n override the direct effect of this
r hyperglycemic agents on the liver
nination of the β cells of the pan-
s been demonstrated to augment
y the hyperglycemic activity of iso-
ol (5) and to reverse the suppres-
pinephrine-induced hyperglycemia
oterenol (4).

periments to be described were de-
) determine the likelihood that iso-
ol-induced insulin release and its
nces in normal rats as well as iso-
ol-induced hyperglycemia in al-
abetic rats are the results of stimu-
adrenergic receptors. Propranolol,
β-adrenergic receptor antagonist,
l to examine these possibilities. In
, the effects of epinephrine and a
lective β-adrenergic drug salbuta-
plasma insulin and glucose levels
o studied. Isoproterenol, salbuta-
nephrine and norepinephrine were
pared for hyperglycemic activity in
liabetic rats controlled with insulin.

ds and materials. Male rats weigh-
oximately 250 g were obtained from
n Rat Company (Madison, Wiscon-
animals had free access to Purina
w and water until the time of the
ntal procedure. Alloxan diabetes
ced by a procedure previously de-
5).

eriments examining the effect of
enol and propranolol on plasma in-
l glucose levels, 10 mg/kg propran-
administered ip 30 min prior to an
of isoproterenol, 1 mg/kg ip or of acidified saline. Animals were lightly anesthetized with pentobarbital (30 mg/kg ip) just before taking blood samples by cardiac puncture. Thirty-five minutes elapsed between the time of injection of isoproterenol and sampling by cardiac puncture. Heparinized blood samples were centrifuged immediately. The plasma was separated and either analyzed immediately or frozen for analysis at a later date (no longer than 2 weeks).

In another set of experiments, epinephrine and salbutamol were examined for their ability to influence plasma insulin and glucose levels in rats. The method of anesthesia required for cardiac sampling and the handling of samples were the same as alluded to previously.

Experiments were also conducted to compare the hyperglycemic activities of isoproterenol, salbutamol, epinephrine and norepinephrine in normal rats and in diabetic rats. Blood samples (100 μl) for glucose determination were obtained by cutting the tip of the tail just before and one hour after ip injection of drugs.

Plasma levels of immunoreactive insulin were determined by means of a modification of the radioimmunoassay procedure described by Hales and Randle (6); insulin binding reagent (Burroughs-Wellcome Co., Research Triangle Park, N.C.) was used instead of individual insulin antibody and precipitating antibody. Glucose levels were determined by the Hoffman procedure (7) as adapted to the Technicon autoanalyzer.

Acidified stock solutions (pH 5) of l-isoproterenol, l-epinephrine, and l-norepinephrine bitartrate were diluted with physiologic saline to appropriate concentrations for injection. Two or three drops of acetic acid were used to enhance the solubility of salbutamol in saline.

Results. As shown in Fig. 1 (left panel) isoproterenol, 1 mg/kg (base) ip caused a

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ISSN 0037-977

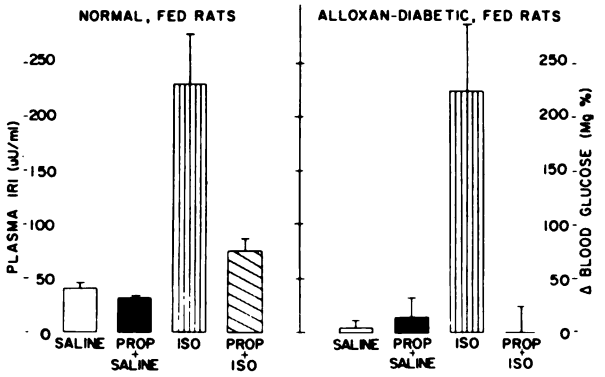

FIG. 1. Effect of pretreatment with propranolol on isoproterenol-induced insulin release in fed (left panel) and hyperglycemia in fed alloxan-diabetic rats maintained with daily injections of pr insulin (right panel). The mean control levels for blood glucose in the diabetic groups (right panel) we 160±50, 163±40, and 176±35, respectively. Each column represents the mean ±SE of six rats. Th between the ISO group and PROP+ISO group in each panel are significant, $P < 0.001$.

marked rise in peripheral levels of immunoreactive insulin at 45 min after injection in fed normal rats. This hyperinsulinemia was accompanied by little change in blood glucose levels (<20 mg/100 ml; see Table II). A 10 mg/kg ip injection of propranolol followed by an injection of saline produced no change in insulin from control. However, pretreatment with propranolol suppressed the isoproterenol-induced change in insulin levels completely (left panel, Fig. 1).

Figure 1 (right panel) summarizes the effects of propranolol and isoproterenol alone and in combination on blood glucose levels in fed alloxan-diabetic insulin-treated rats. Isoproterenol alone produed little or no rise in blood glucose levels in fed normal rats as previously reported (4) and shown in Table II. Interestingly, in rats with few or no functioning pancreatic *beta* cells, isoproterenol became a very potent hyperglycemic agent. The maximum change in blood glucose level produced by 1 mg/kg of isoproterenol in fed, alloxan-treated rats was about 215 mg%. Pretreatment with propranolol eliminated completely the hyperglycemic effect of isoproterenol in diabetic animals. Treatment with propranolol followed by a sham injection of saline did not markedly influence the basal blood glucose levels of diabetic rats.

In another set of experiments the effects of salbutamol and epinephrine on plasma levels of immunoreactive insulin and glucose were investigated in fed, normal rats. As shown in Table I, salbutamol be a more active hyperglycemic a isoproterenol but less active thar rine in fed normal rats. On the ot isoproterenol appeared to be the tent of the two *beta* agonists in insulin release; epinephrine was Salbutamol produced changes o and 95 mg/100 ml in plasma g 0.01, 0.1, and 1 mg/kg ip. Signific tions ($P < 0.05$) in plasma IRI l observed following 0.01 and 1 salbutamol, however, a significant in the insulin/glucose ratio occu with the intermediate (0.1 mg (0.38 compared to 0.23 with s way of comparison and as shown i 0.1 mg/kg of isoproterenol produ sulin/glucose ratio of 0.67 comp value of 0.20 in a paired sali group.

A comparison of the hypergl fects of the adrenergic drugs (T normal and diabetic rats demonst the activity of isoproterenol chang erably more than that of salbuta one dose studied in diabetic rats) rine or norepinephrine when insu was suppressed. Thus, the variatio glucose levels produced by isopro diabetic rats is most probably a re the degree of suppression of insuli

Discussion. The results present paper are in general agreement concept that β-adrenergic agonists

TABLE I. THE EFFECTS OF ISOPROTERENOL, SALBUTAMOL AND EPINEPHRINE ON PLASMA IMMUNOREACTIVE (IRI) INSULIN LEVELS AND PLASMA GLUCOSE LEVELS IN FED NORMAL RATS.

Drug and dose (mg/kg ip)	Plasma IRI (μU/ml)	Plasma Glucose (mg/100 ml)	Ratio IRI/Glucose
Saline (6)[a]	39 ± 5[b]	156 ± 8	0.20 ± 0.02
Isoproterenol (base)			
0.01 (7)	89 ± 20[c]	174 ± 9	0.49 ± 0.10[c]
0.1 (7)	110 ± 19[d]	166 ± 11	0.67 ± 0.15[d]
1.0 (5)	227 ± 44[d]	160 ± 8	1.57 ± 0.29[d]
Saline (6)	31 ± 4	139 ± 4	0.23 ± 0.02
Salbutamol (base)			
0.01 (3)	24 ± 3	157 ± 10	0.16 ± 0.02
0.1 (4)	63 ± 8[c]	173 ± 6[c]	0.38 ± 0.04[c]
1.0 (5)	57 ± 9[c]	234 ± 9[d]	0.24 ± 0.04
Epinephrine (base)			
0.3 (8)	23 ± 4	381 ± 40[d]	0.06 ± 0.01[d]

[a] Number of animals given in parentheses.
[b] Mean ± SE (Students t test used to determine probability values).
[c] $P < 0.05$ Significantly different from control (saline).
[d] $P < 0.01$ Significantly different from control (saline).

TABLE II. CHANGES IN BLOOD GLUCOSE LEVELS INDUCED BY ISOPROTERENOL, SALBUTAMOL, EPINEPHRINE AND NOREPINEPHRINE IN FED NORMAL RATS AND FED ALLOXAN DIABETIC RATS CONTROLLED WITH INSULIN.

Drug and dose (mg/kg ip)	Change in blood glucose[a] (mg/100 ml)	
	Normal rats[b]	Alloxan-treated rats
Saline (5)	17 ± 8	−13 ± 10
Isoproterenol (base)		
0.01 (6)[c]	18 ± 5	57 ± 22[d]
0.1 (6)	10 ± 3	100 ± 9[e]
1.0 (6)	8 ± 4	222 ± 60[f]
Salbutamol (base)		
0.01 (3)	18 ± 10	
0.1 (4)	34 ± 6	59 ± 9[d]
1.0 (5)	95 ± 13	
Epinephrine (base)		
0.03 (4)	13 ± 9	1 ± 9
0.1 (7)	49 ± 11	53 ± 15
0.3 (10)	135 ± 12	99 ± 19[d]
Norepinephrine (base)		
0.1 (6)	22 ± 4	9 ± 17
0.3 (5)	46 ± 12	21 ± 26
1.0 (6)	63 ± 4	71 ± 26

[a] Blood taken from tail 60 min after drug injection.
[b] Fed normal rats serving as controls for comparison with diabetic rats.
[c] Number of animals given in parentheses.
[d] $P < 0.05$.
[e] $P < 0.01$.
[f] $P < 0.001$.

insulin release in the rat, and that α-adrenergic agonists inhibit release (4, 8, 9). Moreover, the data presented here would strongly suggest that isoproterenol has greater insulin *releasing activity* than does salbutamol or epinephrine in the fed normal rat. This suggestion is supported by the evidence that salbutamol and epinephrine have greater hyperglycemic to insulin releasing activity than isoproterenol in the fed normal rats as emphasized by their lower insulin/glucose ratios.

The difference in the metabolic activity between salbutamol and isoproterenol may be attributable in part to the relative rates of metabolic inactivation, salbutamol being inactivated somewhat more slowly than isoproterenol by virtue of its *meta*-CH_2OH (10). In addition, and perhaps more importantly, there may be significant differences in the selectivity of receptor activation between different target organs. Salbutamol is reported to be more of a selective β-adrenergic agonist for receptors in the bronchioles (β_2) than in the heart (β_1) (12). From this study, one might suggest that there is also a difference in selectivity and/or activity at the level of the liver and the pancreas. For example, salbutamol seemed to have a lesser effect on insulin release than isoproterenol and hence, salbutamol was a more active hyperglycemic agent than isoproterenol in normal rats and in normal rabbits (12). Moreover, the insulin-releasing activity of salbutamol may be due in part to its hyperglycemic effect.

It has been demonstrated that isoproterenol evokes dose-related increases in plasma insulin levels in the rat and that these elevations in insulin are strongly influenced by

the state of nourishment (4). Presumably isoproterenol is a weaker hyperglycemic agent in the fed compared to the fasted rat by virtue of its propensity to raise insulin levels more in the fully nourished state. Suppression of insulin released by alloxan treatment was shown to augment the glucose releasing activity of isoproterenol (5) more strongly than that of salbutamol, epinephrine and norepinephrine. That this augmented hyperglycemic effect of isoproterenol in the alloxan diabetic rat is the result of β-receptor stimulation, was demonstrated by the blockade of isoproterenol-induced hyperglycemia by pretreatment with propranolol. Furthermore, the elevations in plasma immunoreactive insulin produced by isoproterenol were also markedly suppressed by previous treatment with the *beta* receptor antagonist, propranolol.

Summary. Isoproterenol-induced insulin release in fed normal rats and hyperglycemia in fed alloxan diabetic rats were inhibited by pretreatment with propranolol. Moreover, salbutamol, another β-adrenergic stimulant, appears to have some insulin releasing activity in the fed normal rat although its glucose mobilizing activity seems to be preponderant. Epinephrine and norepinephrine, on the other hand, have weak or no insulin releasing activity acutely and are more potent hyperglycemic agents than isoproterenol in the fed normal rat. These results suggest that insulin release by isoproterenol and by salbutamol in normal rats can attenuate the hyperglycemic activity of these β-adrenergic receptor agonists.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 341-345 (1977)

n of Factors with Immunosuppressive Activity by a Murine Lymphoblastoid Tumor Cell Line[1] (39667)

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iple interfaces that exist between immune system and cells of pro- growing tumors are currently the much interest. Not only are the ructive or tumor-inhibitory func- ınd B lymphocytes and accessory critically examined, but also var- ne-escape mechanisms of tumor ing elucidated.

ınce that exists, or the relation- een a host and its tumor fre- y be weighted in favor of tumor opposed to immune elimination or cells. For example, the sponta- ase of tumor-specific antigen ells promotes the "blocking" and of T cells at sites anatomically m the tumor (1-4), the forma- locking antigen-antibody com- 7), and the appearance of im- plex-associated tissue pathology ermore, tumor cells have been ecrete factors which possess im- atory properties. A well-docu- mple is the secretion of immuno- products by Ehrlich's ascites tu- *n vivo* (8-11). Similarly, other lines secrete or release antiin- factors (12), factors which in- phage cell function (13), or fac- affect specific T or B lymphocyte 14, 15).

laboratories we have been inter- nmunologic mechanisms which to tumor cell protection, and the ns between immunosuppressive eted by tumor cells of lymphoid origin and factors secreted by normal T and B lymphocytes. We have previously described the secretion by a murine lymphoblastoid tumor cell line (L1210) of several lymphokine-like substances *in vitro* (16). In the present study we describe the release of a lympholine-like factor from an isolated clone of L1210 cells, and the immunosuppressive effects of culture-derived fluids containing such factors.

Methods and materials. Animals. Inbred DBA/2 ♂ and C57B1/6 ♂ mice (18-20 g) and Hartley ♂ albino guinea pigs (0.6 kg) were obtained from Lab Supply Co., Indianapolis, Indiana. All animals were maintained in AAALAC-approved facilities and were handled according to NIH guidelines.

Tumor cells. L1210 lymphocytic leukemia cells (strain LEO8G39) were originally obtained from the Southern Research Institute, Birmingham, Ala. The tumor cell line has been routinely passaged as an ascites tumor in DBA/2 mice, and has also been maintained *in vitro* as a suspension culture in RPMI-1640 medium, pH 7.2, containing 10% heat-inactivated fetal calf serum and 50 μg/ml Gentamicin. All cells were incubated at 37° in a 5% CO_2 in air atmosphere. The clone of cells (L1210/A3) used in the present study was selected as an isolated colony from a culture medium-agarose plate (17), and was subsequently recloned three times at a low cell density in agarose in order to ensure monoclonality. The L1210/A3 cells have since been maintained *in vitro* in suspension culture.

Tumor cell products. L1210/A3 cells were washed in serum-free medium and subsequently recultured in serum-free medium at a cell density of 5×10^6 cells/ml for a period of 24 hr at 37° in a 5% CO_2 in air atmosphere. Following the incubation period, cells were separated from the culture

d by funds from the American Cancer tutional Research Grant IN-111) and the bacco and Health Research Institute and 073).

ddress is the Southern Research Institute, Alabama.

by the Society for Experimental Biology and Medicine

ISSN 0037-977

supernatant fluids (SF) by centrifugation and membrane filtration (0.45 μm pore size). SF were stored frozen (−70°) or were exhaustively dialyzed against distilled water, lyophilized, and stored at 4°; lyophilized SF materials were reconstituted to the concentration desired in phosphate-buffered saline (PBS; pH 7.2) or medium. Protein determinations of SF were done by conventional procedures.

Lymphokine assays. Supernatant fluids were incubated *in vitro* with syngeneic (DBA/2), allogeneic (C57B1/6), or xenogeneic (guinea pig) peritoneal macrophages (PEC) in tests for macrophage migration inhibitory factor (MIF) activity. PEC were harvested from the peritoneal cavities of animals which had received an intraperitoneal injection 5 days earlier of 0.5 ml (mice) or 15 ml (guinea pig) sterile Marcol 52 (Humble Oil Co.). After washing, PEC were packed into plugged capillary tubes which were then cut at the cell-fluid interface. Cell-packed tubes were loaded into Sykes-Moore chambers in which control fluids or medium containing SF were placed (18); serum supplementation of medium containing SF for the MIF tests was 10% fetal calf serum for mouse PEC and 10% guinea pig serum for guinea pig PEC. After a 24–48 hr incubation period at 37°, the areas of PEC emigration from capillary tubes were measured by planimetry, and the migration inhibition values (MI)[3] were calculated. Tests for other lymphokine-like activities in SF were performed as detailed elsewhere (18), in comparison with appropriate controls.

Immunization and SF injection regimens. DBA/2 mice were injected intraperitoneally or intravenously with SF in 0.1 ml PBS prior to, at the same time as, or after, the intraperitoneal injection of 3×10^8 washed sheep erythrocytes (SRBC; in 0.5 ml PBS). For *in vitro* immunization procedures, pooled nonimmune DBA/2 spleen cells were cultured in 35 × 10 mm tissue culture dishes at a concentration of 6×10^6 viable cells in 2.0 ml medium (EHAA, containing 10% fetal calf serum, 5×10^{-5} *M* 2-mercaptoethanol, and 50 μg/ml Gentamicin) at 37°, 5% CO_2 in air for 4 days according to the procedure of Click *et al.* (19); (EHAA containing 2×10^7 washed S with or without SF, was added to culti the beginning of the culture period. control animals and *in vitro* spleen ce tures received an amount of bovine albumin (BSA) equivalent to the amo SF administered to test animals or cu

Plaque-forming cell (PFC) assays. S cells from immunized mice, or splee from the primary *in vitro* antibody re cultures, were assayed for PFC resp specific for SRBC following immuniz The Kennedy and Axelrad modifi (20) of the Jerne plaque techniqu used. Briefly, SRBC monolayers wer pared in 60 × 15 mm Corning tissue c plates by adding erythrocytes to pol sine treated plates; spleen cells, from i nized mice or the *in vitro* cultures, subsequently plated over the SRBC l layers in PBS containing guinea pig se a source of complement. Plates were bated at 37° for 1 hr in order to devel plaques, which were then read macro cally. Indirect (IgG) PFC were enum with the aid of developing antiserum (antimouse IgG, heavy chain specific; pel Laboratories).

Results. Lymphokine assays. Both l and lyophilized-reconstituted L12 culture fluids (SF) exhibited macro migration inhibitory factor (MIF)-like ity *in vitro* with normal syngeneic, a neic, or xenogeneic peritoneal macro cells (Table I); lysates of L1210/A3 ce not possess significant MIF-like activi MI values of ≥80). The MIF-like a associated with L1210/A3-SF was n to cytotoxicity since excessively hig centrations (5000 μg/ml) failed to alt

TABLE I. MACROPHAGE MIGRATION INHIBI FACTOR-LIKE ACTIVITY OF L1210/A3 CULT SUPERNATANT FLUIDS (SF) *in Vitro*.

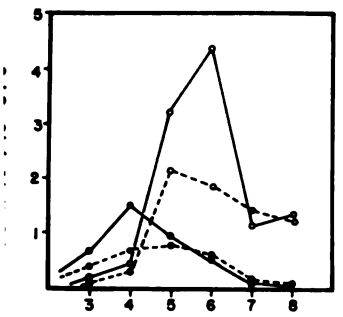
Peritoneal exudate cell source	SF Migratio bition valu SE)[a]
DBA/2 mice (H-2^d)	38 (±
C57BL/6 mice (H-2^b)	43 (±
Guinea Pig	25 (±

[a] SF concentrations = 50 μg/ml; positive c for all experiments were pooled lots of guinea p phocyte culture fluids containing MIF.

[3] MI = [(migration of PEC in SF preparations)/(migration of PEC in cuture medium)] × 100.

y of peritoneal macrophages during a
ıcubation period. The MIF-like activ-
demonstrable with SF preparations
ing as little as 25 μg protein/ml. The
absence of other lymphokine-like
:s, such as proliferation-inhibition
chemotactic factors, lymphotoxin,
'on, and blastogenic factor, was de-
:d by incubation of appropriate tar-
s with SF in comparison with positive
s of antigen or mitogen stimulated
pig lymphocyte culture fluids.

ınosuppressive effects of SF. The in-
of SF in amounts of ≥50 μg up to 3
ior to or on the same day as SRBC
n resulted in a subsequent suppres-
ɜplenic PFC responses to SRBC (Fig.
h the IgM and IgG responses were
ıntly suppressed. Injection of SF by
the intravenous or intraperitoneal
resulted in immunosuppression. SF
1210/A3 cultures were also capable
ressing the splenic PFC responses of
2 related mice (eg., C57B1/6) to
In contrast to the effects of pretreat-
mice with SF, the administration of
r SRBC immunization caused no
in the splenic PFC responses to
The addition of SF to spleen cell
undergoing a primary *in vitro* anti-
ɜponse to SRBC resulted in suppres-
he resultant day four PFC responses
ı; the *in vitro* suppressive effects,



Immunosuppressive effects of MIF-con-
ɜernatant fluids (SF) from L1210/A3 cultures
ɜnic plaque-forming cell (PFC) responses of
ce to SRBC. (——) controls; 50 μg BSA ip 3
to SRBC immunization. (- - -) 50 μg SF ip
or to SRBC. *P* values for peak control re-
ɜmpared to peak experimental responses are
r IgM (●) and <0.001 for IgG (○).

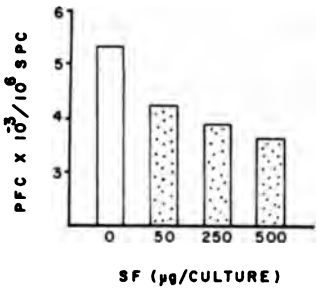


FIG. 2. Immunosuppressive effects of L1210/A3 culture supernatant fluid (SF) preparations on the primary *in vitro* day 4 antibody response of DBA/2 spleen cells to SRBC. The *P* values for suppressor activity of SF preparations of 250 μg/culture or greater are <0.01.

however, were not as marked as the *in vivo* effects, and only the higher concentrations of SF produced significant suppression. SF did not affect the viability of mouse spleen cells *in vitro* at any concentration tested.

Discussion. The L1210 cell is a weakly antigenic, rapidly proliferating lymphoblastoid tumor cell which arose in DBA/2 mice as the result of exposure to methylcholanthrene (21–23). It does not resemble a mature T or B lymphocyte in that it does not possess either membrane-associated Thy 1.2 (θ) antigen or immunoglobulin (24); although this mouse tumor cell does not have C3b receptors on the membrane, several strains of the L1210 cell are reported to possess an Fc receptor for IgG (25). Thus, whether the L1210 cell more closely resembles an immature lymphocyte or a null cell is open to question.

The present study demonstrates that a clone of L1210 cells (L1210/A3) spontaneously releases an MIF-like substance *in vitro* which has macrophage migration inhibitory properties in the conventional MIF test for syngeneic, allogeneic, and xenogeneic peritoneal macrophages. The MIF-like activity of the L1210/A3 culture supernatant fluid (SF) preparations was not due to a toxic effect of SF, as determined by dye-exclusion tests, for macrophages or other cells. This study further demonstrates that L1210/A3 culture SF possessing MIF activity suppress the primary *in vivo* and *in vitro* plaque-forming cell (PFC) responses by syngeneic mouse spleen cells to SRBC; although immunosuppression was statistically

significant *in vivo* and *in vitro*, the *in vitro* responses were less marked. We have found previously that ascites fluid from tumor-bearing DBA/2 mice possesses both the MIF and immunosuppressive properties, but the source of such factors (i.e., tumor cells or host lymphoid cells) remains in doubt (unpublished observations).

Whether the MIF-like factor(s) present in L1210/A3 culture SF preparations are also responsible for the immunosuppressive activity, or whether separate "suppressor factors" are present, is under investigation at this time. Preliminary experiments have shown that both the MIF-like material and suppressor activity cannot be separated by conventional column chromatography techniques; the immunobiologically active fraction has a mol wt in the range of >20,000 to <60,000 daltons.

The relationship of L1210-derived immunosuppressive factors to such factors secreted by other tumor cells (26–30) or, indeed, by normal lymphoid suppressor cells (31) remains to be determined. Several reports link MIF-like activity with an antigen nonspecific suppressor factor secreted by appropriately-stimulated murine lymphocytes (32, 33). The "site" of action of L1210/A3 culture SF may be on the afferent portion of the immune response since administration of SF after antigen exposure does not cause suppression. Also, SF preparations do not suppress the blastogenic responses of murine T or B cell populations *in vitro* to mitogens or antigen (i.e., SF does not possess other lymphokine-like activities such as proliferation inhibitory factor or interferon). Since an effect on macrophages has been demonstrated, SF may alter antigen distribution *in vivo* or antigen uptake *in vitro* by such cells, and may thereby cause the observed suppression. In any event, the effects of SF preparations on specific macrophage functions in both T-dependent and T-independent immune responses must be examined in order to test this hypothesis.

Summary. A clone of L1210 cells has been shown to spontaneously release an MIF-like factor *in vitro*. Culture supernatant fluids containing such MIF activity are immunosuppressive when injected with or prior to an antigenic stimulus in syngeneic *mice, and are* also suppressive to a lesser

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Received November 8, 1976. P.S.E.B.M. 1976, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 346-350 (1977)

In Vitro Depression of Tissue Oxygen Uptake by Liquid Fluorocarbon Pre Artificial Blood (39668)

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Liquid fluorocarbons carry O_2 and CO_2 in large amounts. They give O_2 off to body tissues and carry CO_2 away. These properties have led to the suggestion that emulsions of fluorocarbon in physiologic salt solution might function as artificial blood. Workers, using fluorocarbon emulsions as whole blood infusions or transfusions, or employing these emulsions as perfusates for isolated organs (1-3), have equilibrated the emulsion with very high concentrations of O_2. These concentrations generally approach 100% (760 Torr). High O_2 levels have been used because the solubility of O_2 in fluorocarbon is dependent upon the partial pressure, and it was thought that high O_2 concentrations would permit one to take full advantage of the O_2 carrying capacity of the fluorocarbon. However, no work has been published defining the lower limits of ambient O_2 at which fluorocarbon emulsions might be efficacious in comparison with aqueous plasma expanders which carry very little O_2. Moreover, little data has been published concerning the effects of fluorocarbon emulsions on tissue *in vitro*, in systems much simpler than those employing whole animals or whole organs. The studies described below employ a system to measure tissue oxygen consumption (Q_{O_2}) and to test the effects of a reduction in ambient O_2 on tissue Q_{O_2}, when fluorocarbon emulsions are used as the suspending medium in the tissue bath. We find that fluorocarbon emulsions can depress Q_{O_2}, and the depression is more marked as P_{O_2} is reduced below 760 Torr. In seeking an explanation for this effect, we find that O_2 diffusion falls in fluorocarbon as the P_{O_2} falls, but this phenomenon is not of sufficient magnitude to explain the effect on Q_{O_2} in these particular experiments.

Materials and methods. Microdissection technique. A microdissection tech 5) was used to obtain respirat chioles from adult male Sprague rats (220–225 g), and the oxygen tion of the bronchioles was measu a Cartesian diver microrespirome section of the lungs to obtain the re bronchioles was performed with a croscope containing a calibrated the eyepiece. After determinatio diameter of the respiratory bron *situ*, a tubular length was excise adjacent pulmonary tissue and cu bular segments, each approxima μm in length, 300–400 μm in diam 50–100 μg in dry weight. The ti ments were transferred to a Petri with a physiological salt solutio equal to 7.30±0.05 (KCl 2.8 m*M* m*M*, NaCl 142 m*M*, NaH_2PO_4 11 cose 1.1 m*M*) or a fluorocarbon/pl cal salt solution emulsion. The br consisted of mucosa and smooth m were free of cartilage.

Fluorocarbon emulsions. The f bon emulsions utilized either on fluorocarbons (FC): FC-47 (perflu tylamine or CF_3 $(CF_2CF_2CF_2)_3N$, by 3M Corporation) and PP-5 (per calin or $C_{10}F_{18}$, produced by I.S England).

The emulsions were made in on ways: (a) they were emulsified by s (3) in a salt solution and used aft tion; (b) they were sonicated in water, the emulsion deionized in bed resin, and the physiologic added; (c) they were homogenizec ologic salt solution in a Gaulin nizer at 3000 lbs/in^2. The end poin sification for the sonicates was d passage through a 0.8 μm filter mogenates also were passed throu

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in addition, their optical density as measured and homogenization stopped until the OD had fallen to a

Fluoride ion concentration was d in all emulsions with a specific ion e. It varied from less than 0.5 ppm ppm. Results were independent of ride ion concentration. The emul- ployed 33% to 40% FC on a w/v ie FC was added to Ringers Lactate containing, per liter, 154 mEq Na, , 3 mEq Ca, 109 mEq Cl, 28 mEq To this solution was added 48 g/L of F-68, a nonionic detergent re- or emulsification. The detergent s osmotic strength. The pH was ad- ith Tris-buffer to 7.30±0.10. The ty of the emulsions was 272–292 ., and the results were independent arity over the stated range.

ian diver microrespirometer. The n Diver is a well-established (4, 6–8) for measuring the Q_{O_2} of tissue samples. The samples are a small, floating chamber (the di- se buoyancy is altered as the tissue s oxygen. As O_2 is consumed, the within the system is manipulated in maintain the diver at a fixed level in ation medium. The pressure re- float the diver was plotted against ime, and the change in the amount n in the diver was calculated from derivative of the Ideal Gas Law ect to time to give the oxygen con- of the tissue. All samples showed t change in pressure with time over iod, indicating a steady state condi-

hial tissue, approximately 0.25 μl as suspended in 0.5 μl of the sus- medium (physiologic salt solution carbon emulsion) and placed in the ove the tissue and its substrate was bble which provided buoyancy and source of oxygen. The composition s in the gas bubble could be varied experiments were conducted with ranging from 5% O_2, 95% N_2 to 5% N_2. A KOH seal in the diver orbed all the CO_2 produced by the , 6–8), and there was no change in f the incubation medium measured d after each Q_{O_2} determination.

As distinguished from the existing macro-respirometers, which are equipped with a stirring device, the diver respirometer and in fact all microrespirometers are able to function without stirring, the movement of the gases being considered fast enough to secure equilibrium and adequate mixing in systems of sufficiently small dimensions. Linderstrom-Lang (8) has presented both experimental data and a mathematical analysis which give the limitations of diver dimensions for various gas–liquid systems to ensure adequate mixing and a rapid approach to the attainment of equilibrium. The diver employed in this study was well within the size limitations to ensure adequate mixing in the experiments performed.

In order to ensure that the replacement of salt solution by fluorocarbon emulsion did not alter the mixing properties of the system by creating unstirred layers adjacent to the tissue, the time required for O_2 utilization in the diver to reach equilibrium was recorded. If unstirred layers were present, the time to achieve equilibrium would be altered and/or equilibrium might never be reached. Equilibrium is achieved when the fall in partial pressure of O_2 per unit time becomes constant, and measurements are begun after equilibrium is reached. For rat bronchiole rings of identical size (350 μm diameter) from the same animal, the time to reach equilibrium was rapid and essentially the same in the control physiological salt solution (8.43±0.38 min) and the fluorocarbon emulsions of different dispersed phase (fluorocarbon) concentrations (8.33±0.37) min, 25% (w/v); 8.37±0.31 min, 33% (w/v); 8.57±0.33 min, 40% (w/v).

Diaphragm diffusion cell. The oxygen diffusivity in each of the fluorocarbon emulsions used in the diver was measured using a steady state diaphragm cell which consisted of two compartments separated by a fritted glass diaphragm.

A detailed description of the diaphragm cell can be found elsewhere (9, 10). This experimental technique has been used extensively with good success by other investigators for gaseous diffusion in pure liquids (10), single protein solutions (11), and multiple polymer and protein solutions (12).

Results. Effect of fluorocarbon emulsions on Q_{O_2}. Table 1 shows that the Q_{O_2} was not

TABLE I. BRONCHIOLE Q_{O_2} IN CARTESIAN DIVER MICRORESPIROMETER.

Tissue suspending medium	Fluorocarbon (w/v%)	Q_{O_2}[a] (μL/mg dry wt/hr)	N[c]	Gas mixture (vol %) O_2	Gas mixture (vol %) N_2
Physiologic Salt Solution	–	3.43 ± 0.17	39	20	80
Physiologic Salt Solution	–	2.63 ± 0.11[b]	5	5	95
Physiologic Salt Solution	–	3.51 ± 0.21	5	10	90
Physiologic Salt Solution	–	3.49 ± 0.11	11	95	5
Ringers lactate with F-68 detergent	–	3.39 ± 0.17	5	20	80
Fluorocarbon (PP-5) Emulsion	40	1.69 ± 0.13[b]	10	20	80
Fluorocarbon (PP-5) Emulsion	40	2.71 ± 0.13[b]	5	95	5
Fluorocarbon (FC-47) Emulsion	34	1.99 ± 0.13[b]	5	20	80
Fluorocarbon (FC-47) Emulsion	33	2.69 ± 0.15[b]	10	35	65
Fluorocarbon (FC-47) Emulsion	33	2.98 ± 0.10[b]	10	50	50
Fluorocarbon (FC-47) Emulsion	34	3.23 ± 0.29	10	95	5

[a] Mean ± standard deviation.
[b] Significantly different from control physiologic salt solution at 20% O_2, 80% N_2. $P < 0.01$, Student's t test.
[c] N is the number of bronchioles tested.

depressed in the presence of 20% O_2 and a control solution of Ringers Lactate with detergent. However, fluorocarbon emulsified in the lactate solution, did significantly depress the Q_{O_2} at 20% O_2. As the ambient O_2 was raised, the Q_{O_2} increased, until, in an atmosphere of 95% O_2, the Q_{O_2} in FC-47 emulions was not significantly different from control. Note also that increasing the O_2 in the atmosphere had no effect on the Q_{O_2} of control preparations. Thus, the diffusion of O_2 through the control tissue was not a factor limiting its Q_{O_2}. Moreover, the hourly Q_{O_2} was constant over the entire period of observations (5 hr) for all tissue samples. Although this check is performed to guard against a leaky diver, it also indicates that for tissue in the FC emulsions, suppression of Q_{O_2} was rapid, and thereafter was nonprogressive.

O_2 diffusion in fluorocarbons and fluorocarbon emulsions. The Q_{O_2} data could be explained if O_2 diffusion became impaired in fluorocarbons as O_2 tension fell. To test this hypothesis, we measured the apparent O_2 diffusion coefficient in pure fluorocarbons and in fluorocarbon emulsions, using the steady state diaphragm cell (Methods). Table II shows that the apparent diffusion coefficient for O_2 in the pure fluorocarbons was high at P_{O_2} = 760 torr. The coefficient obtained at P_{O_2} = 760 torr was similar to that for three other pure fluorocarbons published in the literature (13). We have not encountered data at lower P_{O_2}, and the *measurements of this study* show a decreasing O_2 diffusion coefficient with a fall in P_{O_2}. These observations are compatible with the implications of Zander's data (14) showing an increasing fall in solubility coefficient as P_{O_2} fell. The latter implies some chemical binding of O_2 by FC, with an increasing number of chemical binding sites available for O_2 as P_{O_2} falls. These sites would be available to "trap" O_2 and retard its movement through the fluorocarbon down the gradient of O_2 tension. Measures of diffusion in the diaphragm cell would not distinguish this phenomenon from others impairing movement of O_2 molecules. Since the diffusion coefficient will be dependent upon the P_{O_2}, we feel the term "apparent" is best used in describing the coefficient.

Discussion. Although movement of O_2 through FC is impaired as P_{O_2} falls, this change in diffusion coefficient fails to provide a viable explanation for the fall in Q_{O_2} which we observed accompanying the decline in P_{O_2}. Table II shows that at 150 torr, the apparent diffusion coefficient of O_2 in FC and in FC emulsions is higher than the coefficient in H_2O. In addition, we have calculated the flux of O_2 through the diffusion cell in ml O_2/sec and found that even at a P_{O_2} of 50 torr, the flux would exceed that through H_2O at similar P_{O_2}. This is because total flux is proportional both to the diffusion coefficient and to the O_2 content of the fluid. FC carries more O_2 than water, and this factor overrides the reduction in the diffusion coefficient.

Since reduced diffusion of O_2 cannot ex-

TABLE II. O_2 DIFFUSION IN PURE FLUOROCARBONS AND IN FLUOROCARBON EMULSION AT 37°.

Substance	P_{O_2}(mmHg)	D[a]	C_{O_2}* (ml O_2/ml)[b]	N (ml O_2/sec)[c]
FC-47	760	5.79 ± 0.20	0.362 ± 0.019	76.21
FC-47	150	4.97 ± 0.19	0.062 ± 0.003	11.20
FC-47	100	4.35 ± 0.18	0.037 ± 0.002	5.85
FC-47	50	1.93 ± 0.10	0.015 ± 0.001	1.05
25% FC-47 Emulsion	760	3.03 ± 0.09	0.093 ± 0.005	10.27
25% FC-47 Emulsion	150	2.84 ± 0.10	0.018 ± 0.001	1.81
33% FC-47 Emulsion	760	3.21 ± 0.14	0.117 ± 0.008	13.69
33% FC-47 Emulsion	150	2.96 ± 0.09	0.020 ± 0.001	2.15
H_2O	760	2.75 ± 0.12	0.024 ± 0.001	2.38
H_2O	150	2.75 ± 0.12	0.005 ± 0.002	0.46
H_2O	100	2.75 ± 0.12	0.003 ± 0.0001	0.31
H_2O	50	2.75 ± 0.12	0.002 ± 0.0001	0.16

[a] D = diffusion coefficient, mean ± standard deviation × 10^{-5} cm^2/sec.
[b] C_{O_2}* = O_2 content when equilibrated with the stated P_{O_2}, Mean ± Standard Deviation.
[c] N = O_2 "flux", $N = (DA/L)(C_{O_2}^* - C_{O_2})$ where A/L is an apparatus constant, and C_{O_2} is zero.

lain the observed depression of Q_{O_2}, we nust seek elsewhere for an explanation of he findings. There exists the possibility that here is a toxic effect of FC on the tissue. luorocarbons are known to displace structures within liver cells and cells of the reticuoendothelial system (3) and mitochondrial welling has been reported both *in vivo* (3) nd *in vitro* (15).

In vitro mitochondrial respiration has een depressed by FC (15). However, the *in ivo* observations of mitochondrial swelling though not the displacement of cellular organelles) could conceivably be artifact, hile the *in vitro* effects on mitochondria ave been reported only for chlorinated, aseous fluorocarbons rather than the liqids used here. Moreover, bronchial smooth nuscle is the predominant tissue in our ronchial rings, and we are not aware of ublished data describing toxic effects of luorocarbons on smooth muscle. We have, n fact, found only a single report (16) of Q_{O_2} measurements in th presence of FC, *in itro*. This report fails to demonstrate deression of Q_{O_2}, but the data are peculiar in everal respects. Tissue slices from liver, eart, and kidney were employed and were pparently so thick that diffusion of O_2 was miting and basal Q_{O_2} values were only 1% f those found here. The authors were able o raise Q_{O_2} twenty fold simply by increasing he ambient P_{O_2}. An identical effect of inreasing P_{O_2} was found both in the presence r absence of FC. One would expect that Q_{O_2} would improve much more in the presnce of FC since O_2 delivery to the tissue should be increased. Perhaps the slices were so thick that FC at their surface had little impact on their overall Q_{O_2}.

Whatever the reason for the effect we have observed, our data indicate that the depression of Q_{O_2} is gradually overcome as concentrations of O_2 approach 100%. However, the degree of Q_{O_2} depression and the capacity of increasing O_2 tensions to overcome this depression may be dependent upon the fluorocarbon used and its concentration. Our data show that the effect of a PP-5 emulsion on Q_{O_2} was not completely reversed by 95% O_2, while the effects of a slightly lower concentration of FC-47 were reversed by 95% O_2.

Since O_2 tensions approaching 100% are frequently employed with FC systems, this may explain the failure of some workers (1, 2) to observe the effect we have seen after infusion or transfusion of whole animals or organs with large amounts of FC. However, the relationship of our *in vitro* data to an *in vivo* situation remains open to question, since *total* exchange of blood for FC emulsion (2) has been reported in rats with no ill effects (but again at high P_{O_2}). One of us (WIR) has attempted such exchange transfusions in rhesus monkeys, and has succeeded in replacing 50% of the blood at ambient (21%) O_2 tensions, and 75% of the blood while the monkey breathed 100% O_2. Whether the phenomena reported here will provide a barrier to continued development of FC emulsions as a blood replacement remains to be seen. They do suggest that ambient O_2 levels approaching 100% will

have to be maintained as long as high FC levels are circulating.

Sumary. The present studies show for the first time that, *in vitro,* fluorocarbon emulsions depress O_2 uptake of tissue. This depression can be overcome totally or in part by use of O_2 levels approaching 100% of the ambient atmosphere. Our studies also show that the diffusion coefficient for O_2 in fluorocarbons and in fluorocarbon emulsions is dependent upon the O_2 tension and falls as P_{O_2} falls. However, due to the high O_2 content of fluorocarbon, the O_2 flux in fluorocarbons is higher than that in control solutions. Therefore, the fall in diffusion coefficient cannot account for depression of O_2 uptake in these studies. The cause of the depressed Q_{O_2} remains unknown, but toxicity of fluorocarbon should be considered. The data suggest that if fluorocarbon is to be an efficacious blood substitute, high levels of inspired O_2 will have to be maintained as long as high fluorocarbon levels exist in the circulatory system.

Ms. Mary Allgood provided invaluable technical assistance. This study was supported by Grant Nos. HL 16643, HL 14251, and Virginia Lung Association Grants.

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Received September 1, 1976. P.S.E.B.M. 197 154.

SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 351-355 (1977)

nc Deficiency on Compositional Development and Protein Synthesis in Liver, Heart, and Kidney of the Suckling Rat (39669)

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ɜncy causes growth depression animals, perhaps mediated mpairment of normal nucleic tein metabolism (1). Various have examined whether the in- f several different amino acids was depressed by zinc defi- me have obtained results sug- there is an effect on protein 2-4) while others have not ob- ɜnces in response to zinc defi- The conflict in these observa- ɜ explained, in part, by differ- ssues examined, the age of the ɜd, and the severity of the defi-

the nursing dam of adequate n growth retardation (8) and in brain development of the 10). Since zinc deficiency ap- its most severe consequences ıwing tissues, we examined the of [^{14}C]leucine into the pro- heart, and kidney, which are lifferent, although essentially uring the 21-day suckling pe- is provided information about zinc deficiency on growth and development of these organs ıte of incorporation of the la- ıcids into protein.

ınd Methods. Dams of the strain were bred and main- ımmercial laboratory diet until ·tly after parturition, the pups dams were withdrawn, mixed randomly redistributed to the t pups each. The dams were three groups and fed a zinc % sprayed egg white diet (12) ıdified to delete the antibiotic mg inositol/kg diet.[1] The first group, zinc deficient, was fed the diet ad libitum and given distilled/demineralized water to drink. The second group was pair fed on an individual basis with the zinc deficient dams and given 25 mg Zn/l in their drinking water. The third group was given the diet *ad libitum* and the zinc supplemented drinking water.

On the days of analysis (6, 12, or 21 days of age), the pups were injected sc with [U-^{14}C]leucine (0.2 μCi/g body wt). After 20 min, the pups were decapitated, the liver, heart, and kidneys were removed and immediately placed in ice-cold saline. The tissues were weighed and homogenized, and aliquots were analyzed in duplicate for DNA (13), RNA (14), and protein (15), and the incorporation of [^{14}C]leucine was determined by liquid scintillation counting of an aliquot of the sample prepared for protein analysis. Acid-washed glassware was used throughout. Values cited in text represent the means ± standard errors and the significance of the differences was examined by use of Student's *t* test.

Results. The zinc deficient dams developed anorexia and cyclical feeding patterns were observed. By the fifth day, food consumption was significantly depressed (9.6 ± 5.1 g vs 26.4 ± 1.1 g, zinc-deficient vs ad libitum fed control, $P < 0.05$). The anorexia and cyclical feeding persisted during the balance of the experiment and this contributed to the impaired growth of the suckling pups (Table I). The extent of the growth depression increased postnatally since at 6 days of age the zinc deprived pups weighed 78.7% as much as the pups of *ad libitum* control dams, while their body weights were only 53.6% of the *ad libitum*

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he Society for Experimental Biology and Medicine

ISSN 0037-9727

TABLE I. WEIGHT OF SUCKLING RATS ON THE DAY OF ANALYSIS.[a]

	6 days of age		12 days of age		21 days of age	
	weight, g	% *ad lib.*	weight, g	% *ad lib.*	weight, g	% *ad lib.*
Zn def	7.8 ± 0.4[c] (12)	78.7	16.0 ± 0.7[c] (6)	74.2	19.6 ± 0.3[b,c] (6)	53.6
Pr fed	8.3 ± 0.3[c] (12)	82.8	18.8 ± 1.2[c] (6)	87.4	21.7 ± 0.8[c] (6)	59.3
Ad lib	9.9 ± 0.2 (12)		21.5 ± 1.0 (6)		36.6 ± 1.1 (6)	

[a] Values are the means ± the standard errors for the number of animals in parentheses.
[b] Significantly different from pair fed, $P < 0.05$.
[c] Significantly different from *ad lib.*, $P < 0.01$.

controls at 21 days of age. Pups from the pair fed controls tended to grow better than their zinc deficient counterparts, but, their body weights were significantly different only at 21 days of age. Pups of the pair fed controls grew less well than those of the *ad libitum* fed control dams at all ages examined.

The growth of liver, heart, and kidneys in the zinc deficient rat pups also was less than in pups from *ad libitum* fed controls (Table II). Although there was a trend for the organs to be smaller in zinc deficient animals at 6 days of age, only the kidney was significantly smaller when compared with the *ad libitum* fed controls. At 12 and 21 days of age, however, all three tissues were significantly smaller than those from the *ad libitum* fed controls. Tissues from pups suckled by pair fed dams were generally intermediate in weight between values from the zinc deficient and well fed controls, but were not usually significantly different from those of the zinc deficient animals. Compositional determination revealed some differences in response to zinc deficiency and undernutrition (Table II). DNA concentrations were higher in tissues from pups of zinc deficient and pair fed dams and tended to become more disparate with increasing age from the values obtained from pups from *ad libitum* fed controls. This effect was observed in all the tissues, but was most pronounced in the heart. Differences in RNA concentrations did not appear to follow a consistent pattern of responses to either zinc deficiency or undernutrition. Protein concentrations did appear to be elevated in response to zinc deficiency, but only in the liver.

The incorporation of the labeled amino acid into protein appeared to be influenced by the zinc status of the animal, and the *effect was most pronounced* in the youngest animals (Table III). In addition, the liver appeared to be considerably more severely affected than did kidney or heart. At 6 days of age, all three tissues from the pups suckled by zinc deficient dams displayed decreased incorporation of the leucine into protein. This effect did not appear to be due to undernutrition as the pups from pair fed controls did not show the reduced specific activity. At subsequent ages, the livers from the zinc deficient pups continued to display a reduced specific activity when compared with zinc adequate controls (although the difference was not statistically significant from the pair fed controls at 12 days of age). The heart at 12 and 21 days of age did not show differences in specific activity in response to dietary manipulations. The level of incorporation tended to be reduced in the kidneys from zinc deficient pups at 12 and 21 days of age, but the differences were not statistically significant.

Discussion. The dams fed the zinc deficient diet and not supplemented with zinc in the drinking water displayed signs of zinc deficiency including anorexia and cyclical feeding. It has been previously shown that the deficiency experienced by the nursing dams impairs milk production and decreases the zinc content of the milk (8). Pups from such dams display signs of zinc deficiency including reduced plasma and femur zinc and impaired growth (8–10).

The pups in this study also displayed reduced growth and the degree of growth reduction increased with age of the pups and thus with exposure to the deficiency. The growth depression affected the organs examined since they were smaller than those from pups of *ad libitum* fed controls, although the organ weights were consonant with body size and as percentage of body weight did not vary significantly among the

TABLE II. WEIGHT AND COMPOSITION OF LIVER, HEART, AND KIDNEY OF SUCKLING RAT PUPS AT 6, 12, AND 21 DAYS OF AGE IN RESPONSE TO ZINC DEFICIENCY, PAIR FEEDING, OR FEEDING *ad lib.*[a]

	6 days						12 days						21 days					
	Weight (g)	DNA (mg/g)	RNA (mg/g)	RNA/ DNA	Protein (mg/g)	Protein/ DNA	Weight (g)	DNA (mg/g)	RNA (mg/g)	RNA/DNA	Protein (mg/g)	Protein/ DNA	Weight (g)	DNA (mg/g)	RNA (mg/g)	RNA/DNA	Protein (mg/g)	Protein/ DNA
Liver																		
Zn def	0.297	5.454	12.481[b,c]	2.326	157.3[b]	29.2	0.399[b,c]	4.961[b,c]	10.702[c]	2.163[b,c]	162.4[b,c]	32.8	0.668[c]	4.011[c]	9.514	2.397[c]	194.5[b,c]	47.6[b,c]
	±0.024	±0.281	±0.163	±0.150	±3.0	±1.6	±0.020	±0.116	±0.040	±0.048	±2.4	±0.3	±0.023	±0.206	±0.286	±0.098	±12.8	±1.7
Pr fed	0.265[c]	4.891	11.221	2.334	138.5	28.9	0.498[c]	4.577	10.753[c]	2.354[c]	154.6	33.9	0.700[c]	3.976[c]	9.518	2.393[c]	156.8[c]	39.4
	±0.006	±0.271	±0.239	±0.155	±3.2	±2.2	±0.036	±0.083	±0.067	±0.042	±1.6	±0.8	±0.030	±0.078	±0.261	±0.040	±4.7	±0.8
Ad lib.	0.323	5.007	11.475	2.266	148.7	29.3	0.605	4.519	11.461	2.530	151.0	33.4	1.331	3.465	9.317	2.692	132.6	40.6
	±0.005	±0.136	±0.191	±0.056	±4.0	±0.5	±0.030	±0.044	±0.091	±0.032	±3.4	±0.8	±0.038	±0.066	±0.166	±0.053	±1.4	±2.8
Heart																		
Zn def	0.051	5.172	5.296	1.035	115.1	22.4	0.077[c]	5.353	3.853[b,c]	0.722[b,c]	106.4[b,c]	20.2[c]	0.099[c]	3.827[c]	3.188	0.831[b,c]	131.6	34.4[c]
	±0.004	±0.173	±0.318	±0.087	±5.6	±1.4	±0.006	±0.237	±0.164	±0.024	±2.7	±1.4	±0.004	±0.100	±0.104	±0.021	±3.2	±0.6
Pr fed	0.046	5.038	4.713	0.953	104.2	19.2	0.078[c]	5.661[c]	4.543	0.805[c]	121.8	21.6[c]	0.104[c]	3.696[c]	3.327	0.903[c]	132.8	36.1[c]
	±0.003	±0.399	±0.282	±0.054	±5.1	±1.1	±0.005	±0.164	±0.098	±0.026	±2.2	±0.5	±0.004	±0.144	±0.082	±0.018	±3.0	±1.0
Ad lib.	0.060	4.993	5.299	1.065	111.9	22.5	0.103	5.009	4.443	0.890	122.2	24.5	0.187	2.862	3.203	1.21	132.0	46.2
	±0.004	±0.160	±0.141	±0.037	±3.4	±0.6	±0.005	±0.157	±0.080	±0.027	±2.0	±0.7	±0.012	±0.080	±0.078	±0.025	±2.4	±0.8
Kidney																		
Zn def	0.060[c]	9.169	7.413	0.812	101.6	11.1	0.104[c]	9.077	5.537[b]	0.611[c]	106.2	11.8	0.123[c]	9.666[b,c]	5.641[c]	0.585[b,c]	130.9	13.5[b,c]
	±0.004	±0.216	±0.114	±0.029	±2.3	±0.3	±0.004	±0.154	±0.054	±0.012	±2.0	±0.4	±0.003	±0.333	±0.114	±0.015	±5.2	±0.2
Pr fed	0.056[c]	8.833	6.893	0.780	100.0	10.8	0.112[c]	9.508[c]	5.991	0.630	107.7	11.3	0.131	8.648[c]	5.700[c]	0.660[c]	128.9	14.9[c]
	±0.002	±0.410	±0.356	±0.008	±0.5	±0.1	±0.006	±0.196	±0.147	±0.010	±3.5	±0.2	±0.005	±0.166	±0.071	±0.010	±1.3	±0.2
Ad lib.	0.068	9.067	7.120	0.788	100.2	11.1	0.132	8.601	5.536	0.643	102.3	11.9	0.191	8.146	6.054	0.744	136.4	16.7
	±0.002	±0.309	±0.143	±0.018	±1.7	±0.3	±0.003	±0.215	±0.187	±0.011	±3.6	±0.2	±0.007	±0.114	±0.083	±0.005	±2.0	±0.1

[a] Values represent means ± SEM for six animals in each group at each age.

[b] Significantly different from pair fed controls, $P < 0.05$.

[c] Significantly different from *ad libitum* fed controls, $P < 0.05$.

TABLE III. INCORPORATION OF ^{14}C-LEUCINE INTO TISSU
FED OR *ad lib* FED DAMS AT 6

	Liver			
	6 days	12 days	21 days	6 days
Zn def	3.706[b,c]	3.981[c]	4.006[b,c]	1.748[b,c]
	±0.183	±0.111	±0.143	±0.112
Pr fed	5.504[c]	4.002[c]	4.754[c]	2.692
	±0.138	±0.204	±0.209	±0.182
Ad lib.	4.735	4.643	5.339	2.438
	±0.204	±0.083	±0.101	±0.068

[a] Values are means ± SEM for the specific activities in (dpm/mg protein
[b] Significantly different from pair fed controls, $P < 0.05$.
[c] Significantly different from *ad libitum* fed controls, $P < 0.05$.

three groups. No organ system, of those examined, appeared to be selectively damaged in terms of growth.

Effects of zinc deficiency or undernutrition on the composition of the three organs appeared to increase with age of the pups. At 6 days of age, differences between the groups appeared very minimal, but by 21 days of age, the differences were greater and involved all three organs. DNA concentrations were elevated suggesting a reduced cell size and RNA/DNA ratios were reduced perhaps implying a reduction in RNA and protein synthesis. The protein/DNA ratio, frequently used as a measure of cell size, was depressed in both heart and kidney, but was increased in liver. This apparent ambiguity is not readily explained. Ratios of RNA and protein to DNA as indicators of cell size must be interpreted with caution, however, as zinc deficiency has been demonstrated to interfere with the biosynthesis of all of these constituents under various circumstances (1). Possibly, one of the constituents could be affected disproportionately and thus alter the ratio in a way not affecting cell size.

The incorporation of radioactive leucine appeared to be decreased in response to zinc deficiency. In contrast to compositional alterations, the effects on this incorporation were most pronounced at the earlier ages. We observed the greatest depression in leucine incorporation at a time when synthesis of protein by the liver is at a comparatively low level. Protein synthesis appears to be maximal around day 20 and is depressed before day 10 (16). The diminished incorporation, which we observed, could be due to decreased synthesis or to increased degra-

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Received September 1, 1976. P.S.E.B.M. 1976, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 356-359 (1977)

The Influence of Anaerobiosis on Human Erythrocyte Metabolism (39670)

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Incubation of human erythrocytes in a nitrogen atmosphere *in vitro* increases glycolytic rate and 2,3-DPG[1] content (1, 2), changes which have been attributed to the well-known rise in pH that occurs upon deoxygenation of hemoglobin (1). It is not clear, on the other hand, to what extent the absence of O_2 itself contributes to this effect under physiological conditions. In the present study, experiments were conducted in which media buffered at different pH were used to eliminate the pH variable. The results indicate that significant metabolic changes can be ascribed to the absence of O_2 alone.

Methods. Blood was collected from young adult males in acid-citrate-dextrose medium and erythrocytes were separated by centrifugation at 800*g* for 8 min. After the plasma and buffy coat were removed, the packed cells were washed three times with cold Tris-Ringer's medium containing (m*M*): NaCl, 120; KCl, 5.6; $MgCl_2$, 2; Na_2HPO_4, 1; glucose, 10; and tris(hydroxymethyl)aminomethane (Tris, pH 7.40 at 37°), 24. Erythrocyte suspensions with a hematocrit of 25% were prepared with Tris-Ringer's media buffered at four different pH levels; at each pH, the media were titrated so as to cause the pH of anaerobic erythrocytes to match that of aerobic erythrocytes after a given period of incubation. Humidified N_2 was delivered at 8.0 ft^3/hr to deoxygenate erythrocytes and samples were incubated at 37° in a Dubnoff shaker. The N_2-incubated samples were maximally deoxygenated (< 10 mmHg) by 90 min of incubation. Intracellular pH was measured in hypotonic lysates which were prepared by adding 1 ml of packed cells to 2 ml of water. In control studies with oxygenated and deoxygenated erythrocytes, pH values obtained from hypotonic and freeze-thaw lysates were essentially the same. All pH measurements were recorded at 37° with an Instrumentation Laboratory Model 113 pH/gas analyzer.

Protein-free extracts were prepared and standard enzymatic procedures were employed for the measurement of 2,3-DPG (3), FDP, triose-P, 3-PGA, and lactate (4). G6-P, F6-P, PEP, and pyruvate concentrations were determined by a fluorometric procedure (5). Hemoglobin was evaluated by the cyanmethemoglobin method (6). The hemoglobin concentration was greater in cells incubated at alkaline pH (data not shown), signifying the well-known decrease in cell volume as pH is raised (7). Since no difference was noted between hemoglobin concentrations of erythrocytes incubated in air and in N_2 and since the difference between the hemoglobin values at the pH extremes was only 8%, the metabolic data are presented in terms of cell volume. The metabolite profiles (Figs. 1 and 2) were basically the same when the data were expressed in terms of grams of hemoglobin.

Results. Lactate production. When erythrocyte samples were suspended in Tris-Ringer's media that had been buffered to pH 7.70 and 7.40 at 37° and the suspensions were incubated in air (Expt A) and in N_2 (Expt B), respectively, the pH values in both sets of erythrocytes were the same after 90 min of incubation (Table I). After an additional 2 hr of incubation, the final pH values were also identical although they had both decreased by 0.1 unit. Lactate production was 16% higher in the cells incubated in N_2. When a third erythrocyte sample was placed in the same pH 7.70 medium as the oxygenated suspension (Expt A) but was deoxygenated, this suspension (Expt C) had initial and final intracellular pH values

[1] Abbreviations used: 2,3-DPG, 2,3-diphosphoglycerate; G6-P, glucose 6-phosphate, F6-P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; triose-*P*, triose phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PK, *pyruvate kinase.*

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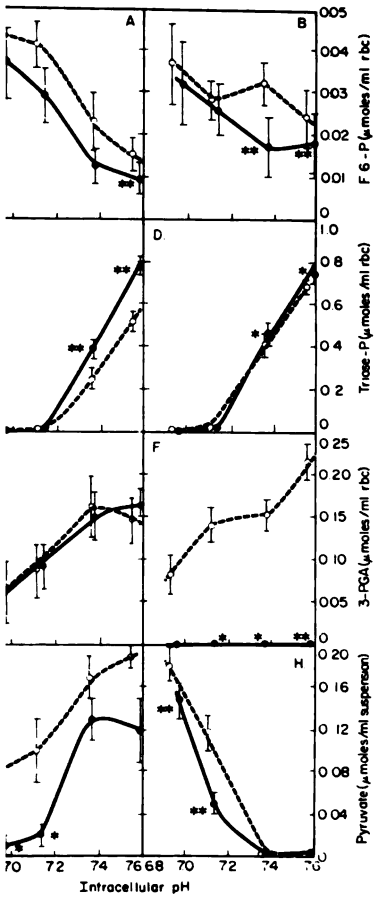
ISSN 0037-9727



ɔncentrations of metabolites in erythro-
d in air (O- - -O) and in N_2 (●——●) as
intracellular pH. Each point is the mean
iments, except for the pyruvate and 2,3-
which are the means of six experiments.
ines bracketing each point represent plus
SEM. *P* values were calculated with the
d experiments. **P* < 0.05; ***P* < 0.01.

0.1 unit greater and a 60%
ate production.

ations of glycolytic intermediates
rs. In a second series of experi-
hrocytes were suspended in me-
fferent pH values so that similar
would be attained in aerobic and
rythrocytes after 150 min of in-
.e., 60 min after deoxygenation
te in the anaerobic erythrocytes.
was raised in the air-incubated
ls, G6-P and F6-P values de-
g. 1A, B) and FDP and triose-P
eased at pH values greater than
7.2 (Fig. 1C, D). These changes have been reported before and ascribed to an increase in phosphofructokinase activity (8). When erythrocytes were incubated in N_2, G6-P and F6-P concentrations were lower and FDP and triose-P concentrations were higher at relatively alkaline pH values where the latter compounds were measurable. This result indicates that phosphofructokinase activity was also accelerated by anaerobiosis at pH values greater than 7.2.

Following deoxygenation, the most marked variation occurred in the 3-PGA concentration, since this compound was undetectible (<0.006 μmole/ml rbc) in N_2 at any pH examined (Fig. 1F). PEP was also de-

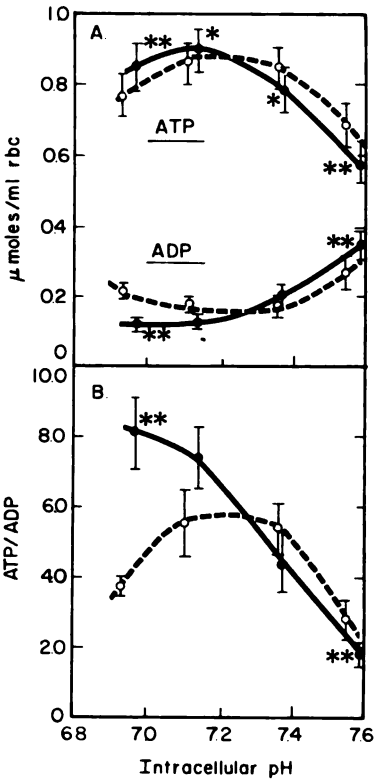


FIG. 2. (A) ATP and ADP concentrations in erythrocytes incubated in air (O- - -O) and N_2 (●——●) as a function of intracellular pH. (B) The ratio of measured ATP and ADP concentrations in erythrocytes incubated in air (O- - -O) and in N_2 (●——●) as a function of intracellular pH. Each point is the mean of six experiments. The vertical lines bracketing each point represent plus and minus one SEM. *P* values were calculated with the *t* test for paired experiments. **P* < 0.05 and **P < 0.01.

TABLE I. INFLUENCE OF ANAEROBIOSIS ON LACTA
CONDITIONS OF CONTROLLED

Expt	Gas phase	Initial
A	Air	7.18 ± 0.01
B	N_2	7.17 ± 0.01
C	N_2	7.26 ± 0.01

[a] Washed erythrocytes were incubated in air (Expt A) Ringer's medium buffered at pH 7.70 and 7.40, respective pH readings were the same after 90 and 210 min of inc incubated in N_2 (Expt C) after they were suspended in the (Expt A). The means ± SEM from three experiments ar
[b] $P < 0.05$, when compared to Experiment A.

pressed (Fig. 1G). These results indicate that pyruvate kinase activity was also accelerated in N_2, causing both PEP and 3-PGA to be decreased. The increase in 2,3-DPG with pH (2) was also observed in our study (Fig. 1E) but no significant change was noted with anaerobiosis.

In air-incubated control cells, pyruvate decreased with increasing pH (Fig. 1H) since NADH availability is greater with increasing glycolytic rate (9). Thus, the lower pyruvate concentrations in N_2 (Fig. 1H) (when these compounds were measurable) were also consistent with a higher glycolytic rate, especially as the latter variation was observed in a N_2 environment (Table 1).

When ATP concentration was plotted as a function of pH (Fig. 2A), bell-shaped curves were obtained which were basically the same variations noted before in the case of the oxygenated cells (10). The curve for deoxygenated cells were located slightly to the left of the curve for oxygenated cells (Fig. 2A), and the graphs for ADP were essentially mirror images of the ATP graphs. The ATP/ADP ratio also displayed a bell-shaped form for oxygenated cells; in contrast, the graph for deoxygenated cells followed a downward course from a value at relatively acid pH, which was higher than that noted for oxygenated cells (Fig. 2B).

Discussion. When erythrocytes were incubated in a N_2 atmosphere after the pH increase associated with deoxygenation had been compensated for, an acceleration of glycolysis was observed which could be attributed to anaerobiosis alone (Table I). The fact that 3-PGA and PEP concentrations decreased under these conditions, instead of increasing as would be expected

: overridden the increased 3-PGA
on by PK. These authors attributed
eases in PK as well as PFK activities
crease in the ATP/ADP ratio (16).
ults (Fig. 2) indicate that the latter
could contribute to the PK activa-
alkaline pH but not at acid pH.
PK effect may be significant because
yme may be modulating carbon flow
the principal and alternate glycoly-
ways. When free 2,3-DPG concen-
declines in N_2, product inhibition of
G mutase decreases (18) and carbon
be diverted toward 2,3-DPG syn-
d binding to deoxyhemoglobin. Al-
this would have a salutary effect on
ery to the tissues (19), ATP synthe-
d be seriously jeopardized. If PK
were also raised, the flow of sub-
rough the phosphoglycerate kinase
may not be reduced and the rate of
nthesis may be essentially normal.
rease in 3-PGA concentration in the
e of increased lactate production in-
that these conditions prevailed in
lies. The ATP concentration, more-
s not seriously altered under anaer-
ditions.

ary. Anaerobiosis caused a small
in lactate production when oxy-
and deoxygenated human erythro-
d similar initial and final pH values.
ange could be attributed to PFK
n. Under physiological conditions,
appeared to be activated, as judged
narked decrease in 3-PGA concen-
When the pH rise associated with
nation was allowed to take place, a
d greater increase in lactate produc-
observed.

This work was supported by USPHS Grant HL-13567.

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Received September 1, 1976. P.S.E.B.M. 1976, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 360-364 (1977)

Maintenance of Glutathione Levels in Renal Cortex Slices of the Rat[1] (39671)

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Glutathione (GSH) is purported to play a central role in the transport of amino acids and perhaps other substances in mammalian kidney. For each amino acid molecule that is transported into a renal tubular cell, a molecule of GSH is broken down and later resynthesized. The synthetic steps require metabolic energy in the form of ATP. The flux of amino acids through kidney is high; accordingly, turnover of GSH is rapid. Maximal rates of degradative and synthetic enzymes of rat kidney are sufficient to catalyze the breakdown and resynthesis of several grams of GSH per day (1).

Kidney slices are frequently used in transport studies (2) but relatively little attention has been paid to GSH levels in the slices. Amino acids released from GSH degradation would not all be expected to be utilized for resynthesis of the tripeptide. Furthermore, considerable leakage of free amino acids from kidney slices is known to occur (2). Thus, GSH levels would be expected to fall off with time in kidney slices being incubated in a medium unfortified with the component amino acids of GSH.

This work was carried out in order to determine how well GSH levels were maintained in kidney cortex slices under standard incubation conditions, and to study the influence of metabolism and added amino acids or protein on GSH levels. In addition, since maleic acid has been shown to depress renal GSH levels *in vivo* (3) and to inhibit renal amino acid transport *in vitro* (4) and *in vivo* (5), the effect of this compound on GSH levels in the kidney slices was studied.

Methods. Animals. Male Holtzman rats of weight range 175-380 g were used in all experiments as the source of kidney tissue. These animals were maintained in an air-conditioned room with an automatic 12 h light/darkness cycle and fed Purina Rat Chow (Ralston Purina Co., St. Louis, Missouri) and water *ad libitum*.

Chemicals. All buffers and solutions were prepared in deionized distilled water and analytical reagent grade chemicals were used except as specified below.

The following chemicals were obtained as the highest purity available: glutathione and L-cysteine (Calbiochem, Los Angeles, California); glycine, L-glutamic acid, and 5,5'-dithiobis-(2-nitrobenzoic acid), (DTNB), (Fisher Scientific, Fair Lawn, New Jersey); bovine serum albumin (Miles Laboratories, Kankakee, Illinois); maleic acid and L-methionine (Eastman Kodak, Rochester, New York); and L-glutamine and dithiothreitol (Sigma Chemical, St. Louis, Missouri). 2,4-dinitrophenol was synthesized in the Department of Nutrition, Harvard School of Public Health.

Solutions added to tissue slice preparations were corrected to pH 7.4 with NaOH or HCl. Cysteine solutions were always prepared immediately before use to avoid oxidation of the sulfhydryl group.

Tissue slices. Renal cortex slices were prepared from rat kidney as described by Rosenberg *et al.* (2). The average thickness obtained with the Stadie-Riggs tissue slicer was 0.50 mm. Each kidney yielded four cortical slices. Prior to incubation, slices were removed from storage in ice-cold

[1] This work was supported by Research Grant No. OH00315 from the National Institute of Occupational Safety and Health and Training and Research Grants Nos. ES00045 and ES00002 from the National Institute of Environmental Health Sciences, U.S.D.H.E.W.

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[3] This study formed a part of a thesis submitted to the Department of Biology, Harvard College, by A. C. Wilder, in partial fulfillment of the requirements for the degree of Bachelor of Arts and Honors in Biological Sciences.

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ISSN 0037-9727

Ringer buffer, gently blotted on noistened filter paper, and weighed ending three slices at a time from a re hung inside a Mettler analytical . The average wet-weight of tissue k (three slices) was 110 mg. After g, slices were placed in 25 ml coniks containing Krebs-Ringer buffer t on ice until the start of the preincunterval.

ations. Slices were incubated at pH her in Krebs-Ringer bicarbonate or Krebs-Ringer phosphate (KRP) ontaining 10 m*M* glucose (6). These were freshly prepared on the day of eriment. Incubations were carried a Dubnoff incubator agitating at 5 oscillations/min. Flasks were cov- an air-tight hood and gassed with $_2$/5% CO_2 when KRB was used, or $)_2$ when KRP was used. In anaerobic ients 95% N_2/5% CO_2 (for KRB) d. Flow rate was adjusted to 4 ft^3/hr, it to produce bubbling from under d. Temperature was maintained at ' except in reduced temperature exits when 25 ± 1° or ice-bath temperas maintained. Unless otherwise d, slices were preincubated at the iture to be maintained in the experior 15 min. Incubations were then ed for 10–120 min in time-course ients or 60 min in single time-point ients. At the end of the incubation , flasks were removed from the incu- an ice bath and the slices quickly d with forceps, dipped briefly into old buffer, blotted with buffer-moistlter paper and placed into micro glass hand-driven homogenizers ing 0.90 ml of cold 5% trichloroace- (TCA) containing 1 m*M* disodium ediaminetetraacetic acid (EDTA). orough homogenization, aliquots of were removed and placed into 10 × test tubes and centrifuged at 1000*g* min to sediment the TCA-precipioteins. Aliquots of the clear super- 0.40 ml) were transferred to 13 × test tubes for assay of GSH.

thione (GSH) assay. The method of and Lindsay (7) was used. The yelor produced by release of 5-thio-2-nitrobenzoate by thiol cleavage of 5,5'-dithiobis(2-nitrobenzoic acid), (DTNB), at pH 8.0 was read at 412 nm in a final volume of 5.00 ml.

Statistical treatment. Glutathione concentrations are expressed as μg GSH/g wet weight of tissue. Data are presented as mean ± SE based on values from three incubation flasks. Significance of differences between means was determined using Student's *t* test, with $P < 0.05$ being regarded as significant.

Results. Time-course of GSH maintenance and effect of added protein. Kidney cortex slices were incubated in KRB buffer at 37° under 95% O_2/5% CO_5 for 30, 60, 90, and 120 min. Glutathione levels in the slices fell during the incubation interval as shown in Fig. 1. Addition of bovine serum albumin (BSA) to the incubation medium at a concentration of 4% (w/v) had no effect on the disappearance of GSH.

Effect of maleate on time-course. Following a 10-min preincubation at 25°, slices were transferred to flasks containing KRB buffer or buffer containing 3 m*M* disodium maleate and incubated at 37° for 10, 20, 30, 60, 90, and 120 min (Fig. 2). Maleate had no effect on the rate of disappearance of GSH between 10 and 60 min of incubation, but GSH levels in maleate-treated slices dropped to lower levels than control slices at 90 and 120 min.

Effect of amino acids and dithiothreitol. Slices were preincubated in KRB buffer at 37° for 10 min before addition of amino acids or dithiothreitol (DTT). Incubation was then continued for 60 min (Fig. 3). Addition of glycine or glutamic acid singly

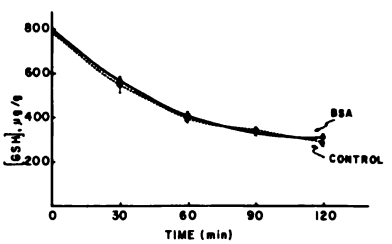

FIG. 1. Time-course of disappearance of glutathione (GSH) from renal cortex slices. Slices were incubated for 30, 60, 90, and 120 min in KRB (control) or KRB containing 4% (w/v) bovine serum albumin (BSA).

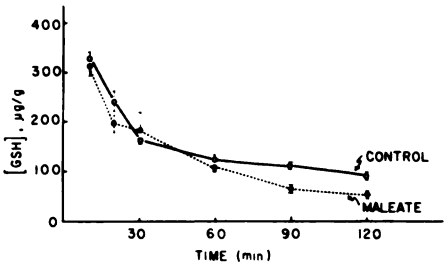

FIG. 2. Time-course of disappearance of glutathione (GSH) from renal cortex slices. Slices were stored at 0° and then preincubated in the same flasks for 10 min at 25°. At zero time slices were transferred to fresh KRB buffer (control) or KRB containing disodium maleate (3 m*M*) and incubated at 37° for 10, 20, 30, 60, 90, and 120 min.

in 0.1 m*M* concentraton did not affect the GSH level. However, adding these amino acids in combination raised the GSH level somewhat above control. The same combination at 3 m*M* concentration produced a marked effect, even though no source of sulfur had been added. Methionine was added at 3 m*M* concentration as a sulfur source, but the result was a depression of the GSH level below the control value. When this amino acid was added to the glutamic acid and glycine combination, the resulting GSH level was slightly lower than with only glycine and glutamate present. Cysteine alone was effective in significantly raising the GSH level and was nearly twice as effective if DTT was included. Dithiothreitol on its own had little or no effect on the GSH level, indicating that DTT acts by preserving the free sulfhydryl groups of cysteine rather than by furnishing sulfur itself. This effect was seen again with the inclusion of all three component amino acids of GSH: the combination was markedly effective, but inclusion of DTT with glycine, glutamate, and cysteine produced the most effective combination tested. Glutamine at 1 m*M* concentration was somewhat more effective than the mixture of 0.1 m*M* glycine and glutamic acid.

Metabolic influences. Table I summarizes the results of three separate experiments testing different aspects of the dependence of GSH maintenance on metabolism. Replacement of oxygen by nitrogen in the incu*bator atmosphere* produced over a twofold drop in GSH concentration. Incubation with dinitrophenol, an uncoupler of oxidative phosphorylation, also resulted in lowering the GSH levels below that of controls. Reducing the temperature to 25° or 0° resulted in successively increasing the GSH level in the slices.

Discussion. Albumin is sometimes added to incubation media as a nonspecific stabilizer of cell membranes (8) but it had no effect on the parameter being measured here. Glutathione disappears rapidly from solutions containing albumin due to the formation of mixed disulfides with the protein (9). If free GSH were simply diffusing out of the slices, a faster rate of disappearance of GSH might have been expected with BSA acting as a trapping agent in the medium. Such was not the case, however.

The effect of maleate on the slice preparations was in marked contrast to the effect on renal GSH *in vivo* (3) when maximal effects on GSH depletion were produced in 30 min. In the *in vivo* situation, however, GSH levels in kidneys of control animals are maintained constant rather than decreasing as in the slice preparation. Glutathione depletion *in vitro* may be difficult to achieve unless the conjugation product is constantly removed from the medium (it is excreted in the urine *in vivo*) since some GSH-conjugating en-

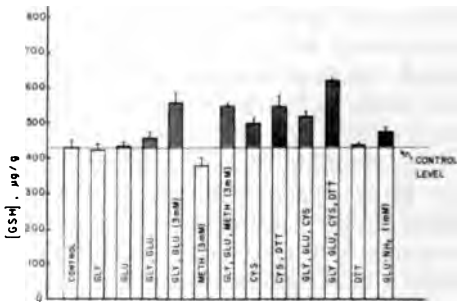

FIG. 3. Effect of amino acids and dithiothreitol on glutathione (GSH) levels in kidney cortex slices. Substances were added to KRB medium at zero time following a 15 min preincubation interval and incubation continued for 60 min. Control = buffer only; GLY = glycine; GLU = glutamic acid; METH = methionine; CYS = cysteine; DTT = dithiothreitol; GLU-NH_2 = glutamine. Except when indicated inside the bars, final concentration of each added component was 0.10 m*M*. Shaded portion of bar indicates net increase of GSH concentration over mean control level.

TABLE I. METABOLIC INFLUENCES ON GLUTATHIONE (GSH) LEVELS IN KIDNEY SLICES.[a]

Oxygen[b]		Temperature,[c] °C			Dinitrophenol[d]	
Present	Absent	37	25	0	Absent	Present
460 ± 14	211[e] ± 7	310 ± 20	650[e] ± 40	770[e] ± 70	370 ± 5	280[e] ± 10

; of three separate experiments. Renal cortex slices prepared as outlined in Methods and incubated for
ıe appropriate buffer system following a 15-min preincubation.
uffer, 37°, under 95% O_2/5% CO_2 or 95% N_2/5% CO_2.
:d KRP buffer containing 0.11 *M* $CaCl_2$, under 100% O_2.
rd KRP buffer, under 100% O_2 at 37°. Dinitrophenol (final concentration 1.0×10^{-4} *M*) added at
f preincubation interval.
antly different from each respective control ($P < 0.01$).

: inhibited by their products (10).
ults obtained with the amino acids
that the GSH lost by degradation
at least partially replaced by sup-
ıino acids for resynthesis. Gluta-
glycine are utilized in a number of
other than GSH synthesis. It was
: that GSH levels could be boosted
ing only these amino acids in the
even though cysteine was not sup-
ese results have considerable sig-
in studies of amino acid transport.
; glutathione levels can increase
iveness of amino acid transport by
amyl cycle (1). Thus, increasing
ntration of one of the component
ds of GSH could increase its rate
rt through its effect on the level of
ıtained in the slice.
tabolism experiments verify first
the enzymic degradation of GSH
anaerobically whereas synthesis
oxidative metabolism. These ex-
also discount the possibility that
ppears from the slices due simply
ɔn of the SH group. Finally, cool-
slices would be expected to de-
radative and synthetic processes
hus preserving initial levels of
vas observed.
ıis work was in progress, Hewitt *et*
ported the same steady decline in
ls in kidney slices as reported
y were able to drastically accelera-
e of the disappearance by treating
with high concentrations of diam-
ective agent for the intracellular
of GSH. Amino acid transport
ımitantly inhibited in the treated

ven though GSH levels may sim-
ply reflect the overall metabolic state of a slice preparation, the existence of relatively specific inhibitors of GSH metabolism and the possible direct interaction between amino acid transport and GSH suggest that GSH levels should be regarded as an index of viability in kidney slices with respect to transport.

Summary. The level of reduced glutathione (GSH) in renal cortex slices was shown to be a function of overall metabolism and availability of the component amino acids of GSH. Incubation of slices in oxygenated Krebs-Ringer media at 37° resulted in a steady loss of GSH. Nearly 50% of the initial GSH was lost in 1 hr. This loss was offset when the incubation was carried out a reduced temperature. Anaerobic conditions or incubation with dinitrophenol enhanced the disappearance of GSH. Fortification of the medium with glycine and glutamic acid at 3 m*M* concentration each or glycine, glutamic acid and cysteine each at 0.1 m*M* resulted in a marked increase of GSH levels after 60 min incubation compared to unfortified slices. Dithiothreitol enhanced the effect of cysteine, but had no significant effect on its own. The presence of 4% (w/v) bovine serum albumin in the medium had no effect on loss of GSH. Disodium maleate had no effect for up to 1 hr of incubation and produced only slight additional loss of GSH after 1.5 and 2.0 hr of incubation.

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Received September 7, 1976. P.S.E.B.M. 197 154.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 365-367 (1977)

Murine Tumor Sera on Adsorption of IgG-Sensitized Erythrocytes by Murine Sarcoma Tissue[1] (39672)

LAW P. TARGOWSKI, C. JOHN ABEYOUNIS, AND FELIX MILGROM

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reports have shown that sera from earing tumors (1) and from human vith various types of neoplasms (2) nmune complexes. Such immune s can bind specifically to tumor *itro* and block cytotoxicity of im- ıphocytes (1). Evidence also has ›vided, by this laboratory and ıat immune complexes can bind ifically" in that sensitized sheep tes adsorb to tumor tissue via the n of the IgG molecule (3, 4). We wn recently that immune com- ımposed of soluble antigens and esponding antibodies can also bind ifically" to the Fc receptors of lls, as well as to those on spleen The binding of these complexes y inhibited adsorption of antibody- erythrocytes by these tissues. We ;idered that perhaps immune comnposed of tumor antigen and anti- sera from tumor-bearing animals › bind "nonspecifically" to the Fc of tumor cells. Accordingly, in the tudy, sera from mice immunized ;enic tumor were tested for their to inhibit hemadsorption to Fc of syngenic and allogenic tumor spleen tissues.

ls and methods. Animals. C3H/ ł C57BL/6 mice, 8-12 weeks old, ;t Seneca Laboratories, West Sen- were used for all the experiments.

tissue. Methylcholanthrene-in- rcomas, MCSa-22 and MCSa-3, inally produced in this laboratory aneous injection of the carcinogen 'HeHa and C57BL/6 mice, respec- ıese tumors were maintained by transplantation in the strain of origin. Tumor tissue was usually collected within 2-3 weeks after transplantation. If the tissue was not used immediately, it was stored at −70°. Histopathological examination of paraffin-embedded sections, as well as of cryostat sections, were performed for each tumor specimen tested. Cryostat sections for histopathological examination were taken randomly from sections cut for each hemadsorption experiment. Only neoplastic tissue free from necrosis and inflammatory cell infiltration was used in this study.

Immune sera. C3H/HeHa and C57BL/6 mice were immunized with syngenic tumors by subcutaneous implantation with a trocar of a 3 × 30 mm plug of minced tumor tissue. In 2-3 weeks, when the tumor attained 2-2.5 cm in diameter, it was excised. Four days later, the mice were inoculated again with the same dose of minced tumor tissue. Again, the tumors were excised in 2-3 weeks, and 4 days later, the entire group was inoculated for a third time. The mice were bled 3-5 days after the third inoculation of tumor.

All sera were placed at 4° and were tested for inhibition of adsorption of sensitized sheep erythrocytes within 24 hr. Some of these sera were retested after storage at −70°. However, repeated freezing and thawing of these sera was avoided.

Hemadsorption and inhibition of hemadsorption. Hemadsorption by cryostat-cut tissue sections was performed as previously described (3, 5). Briefly, 10 μm thick tissue sections were placed on a coverslip and dried at room temperature. Wells of microculture slides were filled with a suspension of sheep erythrocytes sensitized by a selected subagglutinating dilution of a rabbit anti-sheep erythrocyte serum. The wells were sealed with the coverslips in such a way that the tissue section was submerged in the suspension of sensitized erythrocytes. The

ted by Grant No. IM-24A from the Ameri- Society and the Richard W. and Mae Stone te.

' by the Society for Experimental Biology and Medicine

ISSN 0037-9727

preparations were incubated, coverslip down, for 1 hr at room temperature. Then they were turned, coverslip up, and left at this position at room temperature to allow unattached erythrocytes to fall down from the tissue. The sections were then examined microscopically at low magnification.

Adherence of sensitized sheep erthrocytes to the sections was recorded as a positive hemadsorption test. If a tissue section was completely covered by sensitized sheep erythrocytes, the test was graded as 4+. A reaction was considered 2+ if approximately half the tissue was covered by erythrocytes. Sparsely attached erythrocytes on the tissues was considered as a 1+ reaction. Absence of erythrocytes from the tissue was recorded as a negative result.

Inhibition studies were performed by incubating tissue sections in a moist chamber at room temperature for 20 min with various sera or with phosphate buffered saline, pH 7.2 (PBS). Subsequently, the tissue sections were washed three times in PBS and the hemadsorption test was performed. In order to avoid experimental variation, all data presented in each of the tables were obtained on the same day.

Results. Sera from 72 C57BL/6 and 68 C3H/HeHa mice immunized with the syngenic tumors were tested for inhibition of hemadsorption. Virtually all sera tested gave inhibition. An example of these results is shown in Table I, in which the immune serum inhibited hemadsorption up to a dilution of 1:40, whereas normal serum did not inhibit even at a dilution of 1:10. It should be noted that inhibition was also obtained with immune serum heated at 56° for 30 min. None of 72 sera from normal C57BL/6 and C3H/HeHa mice gave inhibition.

TABLE I. ADSORPTION OF SENSITIZED SHEEP ERYTHROCYTES BY C57BL TUMOR TISSUE. INHIBITION BY SERUM FROM A NORMAL C57BL MOUSE AND A C57BL MOUSE IMMUNIZED WITH SYNGENIC TUMOR.

	Serum from:			
Dilution of serum 1 to:	Normal mouse	Immunized mouse	Heat inactivated serum from immunized mouse	Saline
10	2+	–	–	
20	3+	–	–	3+
40	3+	–	–	

The specificity of inhibition of the h sorption with sera from mice immu with syngenic tumors was also investi An example of such an experiment is in Table II. Immune sera from C3H n well as those of C57BL mice inhibited adsorption by both C3H and C57B mors. As seen in Table III, similar i

TABLE II. ADSORPTION OF SENSITIZED SH ERYTHROCYTES BY TUMOR TISSUE FROM C3H C57BL MICE. INHIBITION BY SERA FROM NO C3H AND C57BL MICE AND C3H AND C57BL IMMUNIZED WITH SYNGENIC TUMOR.

		Hemadsorption mor from	
Sera from	Dilution 1 to:	C3H	C5
Immunized C3H	10	–	
	20	–	
	40	–	
Normal C3H	10	3+	3
	20	4+	4
	40	4+	4
Immunized C57BL	10	–	-
	20	–	-
	40	–	1
Normal C57BL	10	3+	3
	20	4+	4
	40	4+	4
Saline		4+	4

TABLE III. ADSORPTION OF SENSITIZED SHE ERYTHROCYTES BY NORMAL SPLEEN TISSUE FR C3H AND C57BL MICE. INHIBITION BY SERA F NORMAL C3H AND C57BL MICE AND C3H A C57BL MICE IMMUNIZED WITH SYNGENIC TUM

		Hemadsorption spleen from	
Sera from	Dilution 1 to:	C3H	C57
Immunized C3H	10	–	-
	20	–	-
	40	1+	-
Normal C3H	10	3+	3+
	20	3+	3+
	40	3+	3+
Immunized C57BL	10	–	-
	20	–	-
	40	1+	1+
Normal C57BL	10	2+	2+
	20	3+	3+
	40	3+	3+
Saline		3+	3+

results were obtained when normal n tissue from C3H and C57BL mice used in place of tumor tissue.

scussion. Previous work in this labora- (3) showed that murine methylcholan- ıe-induced sarcomas (MCSa) adsorb p erthrocytes sensitized by IgG but not nsitized erythrocytes or erythrocytes tized by IgM antibodies. All these re- were fully confirmed at the beginning e present study.

ıis study demonstrated that sera from immunized with syngenic MCSa in- ed the attachment of sensitized sheep ırocytes to MCSa. In contrast, sera normal animals did not inhibit hemad- tion. The inhibition of hemadsorption ıe immune sera could have been due to ing of tumor-specific antibody to the :sponding antigen on the tumor cell, h might have been identical to the Fc ɔtor or in such close proximity to them render the receptor inaccessible. Alter- ely, immune complexes composed of ɔr antigen and its corresponding anti- could bind "nonspecifically" to the Fc ɔtors of the tumor cells. The latter pos- ty is most likely since we have shown in evious study (5) that BSA rabbit-anti- complexes may bind to Fc receptors of ne tumors and, consequently, inhibit adsorption. Furthermore, in this study, of tumor-bearing mice inhibited hem- rption by allogenic, as well as syngenic ɔrs. In addition, inhibition of hemad- tion was demonstrated with these sera ormal spleen tissue, which obviously do ɔear tumor-specific antigens.

eliminary studies with Raji cell tech- e (6) (not reported in the results) were istent with the described results in that 1 tumor sera tested, all contained im- e complexes detectable by this tech- e.

It could be argued that complement receptors, as well as Fc receptors, could be involved in the hemadsorption phenomenon, and its inhibition by immune murine sera, since tumor cells are known to bear complement receptors (7). Participation of complement receptors was excluded, however, by the finding that inhibition of hemadsorption was not altered by heat-activation of the inhibiting sera.

Summary. Previous work in this laboratory has shown that the adsorption of IgG antibody-sensitized erythrocytes by tumor and normal spleen tissues can be inhibited by immune complexes and heat-aggregated IgG. The present study showed that sera from mice immunized with syngenic tumor cells inhibited adsorption of sensitized erythrocytes by tumor and spleen tissues. These sera inhibited also hemadsorption by allogenic tumor tissue and, significantly, by syngenic as well as allogenic spleen tissue.

These results support the contention that sera from tumor-bearing animals contain immune complexes which block the Fc receptors of tumor and spleen cells indiscriminately.

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Received July 26, 1976. P.S.E.B.M. 1976, Vol. 154.

Increased Thymidylate Synthetase Acti\ Novikoff Hepatom

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The fluorinated pyrimidines, including 5-fluorodeoxyuridine (FdUrd), produce objective responses against a variety of human (1) and experimental (2) neoplasms. However, the development of clinical resistance is a major limitation of these drugs (3). Resistance to FdUrd can be induced in Novikoff hepatoma cells by continued exposure to minimally toxic concentrations of the analog (4). The persistence of FdUrd resistance, even after the cells had been grown for at least five generations in the absence of the drug, established the genetic basis for this phenotypic change. The development of FdUrd resistance was accompanied by the loss of thymidine kinase activity (4), the enzyme which is necessary for the conversion of FdUrd to its active metabolite, 5-fluorodeoxyuridylate (FdUMP) (5).

Several enzymes, including thymidine kinase (6), thymidylate synthetase (7), deoxycytidylate deaminase (7), and ribonucleotide reductase (8), seem to be regulated in concert according to the cell's requirements for DNA synthesis. The interrelationships of these enzymes are shown in Fig. 1. In order to see if the deletion of one of these coordinately controlled enzymes would affect the levels of the others, we compared the levels of these enzymes in FdUrd-sensitive (thymidine kinase-containing) and FdUrd-resistant (thymidine kinase-deficient) Novikoff hepatoma cells.

Materials and methods. Cells and media. The Novikoff hepatoma was originally induced as a solid tumor in rats fed 4-dimethylaminoazobenzene (9). The transplantable tumor was subsequently grown in the ascitic form, from which the tissue cultures lines, N1-S1 (thymidine kinase-containing, FdUrd sensitive) and N1-S1/FdUrd (thymidine kinase-deficient, FdUrd resistant), were de-

[1] This research was supported by grants from the USPHS, National Cancer Institute, CA 14762 and CA *17246.*

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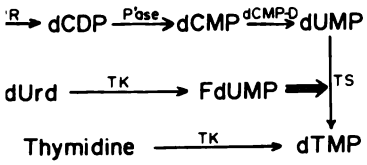
medium 77 and calf serum were from Grand Island Biological (Grand Island, N.Y.) and Inter-ɔcientific Industries, Inc. (Cary, ɔctively. Pluronic F-68 was a gift yandotte Chemical Company te, Mich.). The nucleosides and s used in these studies were pur-ɔm Sigma Chemical Co. The ^{14}C-ɔeled compounds were purchased ν England Nuclear and Amer-rle.

The levels of the enzymes, thymi-e, thymidylate synthetase, deoxy-deaminase and ribonucleotide re-N1-S1 and N1-S1/FdUrd cells are 'able I. The deficiency of thymi-se activity seen in the N1-S1/ lls is similar to that reported by). There was little difference be-S1 cells and N1-S1/FdUrd cells in of deoxycytidylate deaminase or ɔtide reductase. However, there nificant increase in the level of ɔe synthetase activity in the N1-cells.



'athways for interrelating enzymes in-ɔxyribonucleotide metabolism. The abbre-l are as follows: ribonucleotide reductase, ytidylate deaminase, dCMP-D; thymidyl-se, TS; thymidine kinase, TK; and phos-se. The heavy arrow (⟹) indicates inhib-idylate synthetase by FdUMP.

The sensitivities of the thymidylate synthetase from the N1-S1 and N1-S1/FdUrd tumor cells to inhibition by fluorodeoxyuridylate (F-dUMP) were the same. This was determined at various concentrations of F-dUMP, under conditions of equal protein concentrations and under conditions in which the enzyme activities were equal. At equal protein concentrations, F-dUMP, at a final concentration of 5×10^{-8} *M* inhibited the thymidylate synthetase activity in both the N1-S1 and N1-S1/FdUrd cell-free extracts greater than 98%. The cell-free extract from the N1-S1/FdUrd cells was diluted with a solution of 1% albumin to contain approximately the same amount of enzyme activity per unit volume as the cell-free extract from the N1-S1 cells. Under these conditions, FdUMP (1×10^{-8} *M*) inhibited the thymidylate synthetase activity in the N1-S1 and N1-S1/FdUrd cells by 89 and 87%, respectively.

Although its relationship to DNA synthesis is less direct than that of the enzymes listed in Table I, $(Na^+ + K^+)$-ATPase activity appears to be required for the increased DNA synthetic rate which characterizes the blastogenic response of lymphocytes following phytohemagglutinin stimulation (20). The levels of this enzyme in the N1-S1/FdUrd cells are given in Table II. Also shown are the levels of 5'-nucleotidase, which is a plasma membrane-associated enzyme with no known relationship to DNA synthesis. The specific activity of $(Na^+ + K^+)$-ATPase in N1-S1/FdUrd cells was only one-fifth that present in N1-S1 cells, whereas there was little difference in the 5'-nucleotidase activity.

BLE I. ENZYME LEVELS IN CELL-FREE EXTRACTS FROM N1-S1 AND N1-S1/FdUrd CELLS.

nent	Cell density[b] (× 10^{-6} cells/ml)	Enzyme activity[a] TK	dCMP-D (nmoles/30 min /mg protein)	TS	RR
	1.14	16.0	125	1.99	0.150
Jrd	0.84	0.17 (0.01)[c]	174 (1.39)	9.47 (4.76)	0.185 (1.23)
	0.50	23.0	186	4.73	0.125
Jrd	0.70	0.15 (0.006)	224 (1.20)	11.11 (2.35)	0.132 (1.06)

ɔreviations used are: TK, thymidine kinase; dCMP-D, deaminase; TS, thymidylate synthetase; RR, le reductase.

ɔsity of cultures from which enzyme extracts were prepared.

ɔbers in parentheses are the ratio of enzyme levels in the F-dUrd resistant cells to the levels in the F-ɔ cells.

TABLE II. LEVELS OF $(Na^+ + K^+)$-ATPase AND 5'-NUCLEOTIDASE IN THE PLASMA MEMBRANES OF N1-S1 AND N1-S1/FdUrd CELLS.

Experiment	Cell density[a] ($\times 10^{-6}$ cells/ml)	Enzyme Activity ATPase (nmoles of Pi/hr/mg protein)	Enzyme Activity 5'-Nucleotidase (nmoles of Pi/hr/mg protein)
I			
N1-S1	1.14	2.2	–
N1-S1/FdUrd	0.84	0.39 (0.17)[b]	–
II			
N1-S1	0.50	1.03	0.38
N1-S1/FdUrd	0.70	0.20 (0.19)	0.46 (1.21)

[a] The cell density of cultures from which the membrane fraction was prepared (30,000*g* sediment).
[b] The numbers in parentheses are the ratio of the enzyme levels in the resistant cells to the levels in the sensitive cells.

Discussion. The data in Table I show that thymidine kinase activity can be greatly suppressed without causing a concomitant decrease in other enzymes which are usually coordinately regulated with thymidine kinase. This suggests that the mutation which is responsible for the decreased thymidine kinase activity resides in the structural gene for thymidine kinase rather than in a regulatory gene.

The elevated thymidylate synthetase activity in the N1-S1/FdUrd cells may be an adaptive response which allows the *de novo* pathway of dTMP biosynthesis to compensate for decreased dTMP production via the salvage pathway in the thymidine kinase-deficient cells. Alternatively, the elevated levels of thymidylate synthetase present in the N1-S1/FdUrd cells may be an essential part of the FdUrd resistance. The inhibition of thymidylate synthetase activity by FdUMP in cell-free extracts from both N1-S1 and N1-S1/FdUrd cells was similar, indicating that the resistance of N1-S1/FdUrd cells was not due to an altered enzyme which was insensitive to FdUMP. However, the absolute level of residual thymidylate synthetase activity remaining in the resistant cell line after drug therapy (F-uracil or F-deoxyuridine) may be sufficient to allow for nearly normal growth rates. Increased thymidylate synthetase activity has also been reported in cells which are resistant to methotrexate (21), another drug whose primary intracellular target appears to be in the formation of thymidylate.

The significance of the decreased level of $(Na^+ + K^+)$-ATPase activity in the N1-S1/FdUrd cells is unknown. Since the levels of 5'-nucleotidase activity are not significantly different in the two cell lines, the decreased levels of $(Na^+ + K^+)$-ATPase in the N1-S1/FdUrd cells do not appear to be the result of a generalized loss of membrane-associated enzymes. It may, therefore, represent a rather specific alteration in plasma membrane function, possibly affecting nucleoside transport in some manner that decreased the potency of the nucleoside analog. Although rigorous experiments capable of differentiating transport *per se* from uptake have not yet been carried out, preliminary studies show that the N1-S1/FdUrd cells consistently incorporate radioactive ribo- and deoxyribonucleosides into both the acid-soluble and acid-insoluble fractions at a significantly lower level than that observed in the N1-S1 cells.

The multiple enzymatic differences which exist between the N1-S1 and N1-S1/FdUrd cell lines illustrate that the development of drug resistance may be a complex process, involving more than one enzyme locus. These results also emphasize the need to exercise caution when utilizing known genotypic differences between closely related cell lines as an experimental tool, since it is quite likely that unknown differences may easily lead to incorrect conclusions.

Summary. A fluorodeoxyuridine-resistant Novikoff hepatoma cell line, which had been previously characterized as being thymidine kinase deficient, was shown to have elevated levels of thymidylate synthetase. The thymidylate synthetase activity in the fluorodeoxyuridine-resistant cell line was

sensitive to inhibition by FdUMP to the same extent as the fluorodeoxyuridine-sensitive cell line.

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Received July 23, 1976. P.S.E.B.M. 1976, Vol. 154.

Change in Rats' Serum Triglyceride Concent and Exercise

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In 1964, Holloszy *et al.* (1) demonstrated that exercise had a significant effect on lowering serum triglyceride (TG) levels in middle-aged men. Subsequently, other investigators reported similar findings in exercised men (2–5) and rats (6–11). However, other investigators have also reported that exercise has no effect on (12, 13) or increases (14–16) the concentration of plasma TG. Thus, the literature regarding the effects of exercise upon serum triglyceride is confusing and often contradictory (5, 10).

In horses (17) and in rats (18–20), exercise is known to increase thyroid secretion rate. It has long been known that thyroxine plays a significant role in the regulation of lipid metabolism (21, 22). We have shown that the intact thyroid gland is necessary for the hypocholesterolemic effect of exercise (23, 24).

In view of the fact that high lipid concentration in serum is a risk factor associated with increased incidence of premature coronary heart disease (38–41), we investigated one possible means of lowering the serum triglyceride level. We examined the role of the thyroid gland on the hypotriglyceridemic response to physical exercise.

Materials and Methods. Experimental design. The study was designed as a 4 × 5 analysis of variance factorial experiment in which each of the 20 treatment combinations contained 12 male rats (Sprague–Dawley–Rolfsmeyer) weighing 220 ± 20 g at the initiation of the experimental period.

Thyroid groups. Five different thyroid states were produced. Four groups of surgically thyroidectomized male rats were given daily replacement therapy of either saline or L-thyroxine (L-T_4): (i) no L-T_4 replacement (saline injection); (ii) 0.5 μg of L-T_4/100 g body wt; (iii) 1.0 μg of L-T_4/100 g body wt; and (iv) 3.5 μg of L-T_4/100 g body wt. A

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e Lab-Blox) were provided *ad libi-*

m analysis. At the end of the experi- period, the rats were fasted for 12- bled via the thoracic aorta, and then At this time, a postmortem check for ıing thyroid tissue was performed in ırgically thyroidectomized animals. ra was analyzed for TG content (27).

ılts and discussion. Analysis of vari- 28) indicates that the main effect of l status (A), the main effect of physi- ivity (B), and the AB interaction had ly significant influence ($P < 0.0005$) serum TG level (Table I). Analysis of ınce indicates that the varying feed (Fig. 1) of the 20 groups was not an ant factor in influencing the serum ncentrations when the animals were asal diet. This suggests that the serum vels represent primarily endogenous he unadjusted means were used when ıining the effects of the thyroid status ysical exercise on serum TG levels.

ct of thyroxine. When compared to ntrol animals with intact thyroids, se- Gs of the 0, 0.5, 1.0, and 3.5 μg of L- ıted groups were found to be signifi- lower ($P < 0.0001$). This difference ım TG levels may be related, in part, ly weight. Figure 2 shows the final weights of the various treatment . When compared to the control rats, ctive of the level of physical activity, the 0 μg of L-T_4-treated group (athyroid group) had the lowest body weights and, generally, the lowest serum TG concentrations, as compared to the other groups. A similar trend is seen in the 0.5, 1.0, and 3.5 μg of L-T_4-treated groups, i.e., their body weights were lower than those of the control rats with intact thyroids, and their serum TG levels were also lower than the levels of the control groups.

Effect of exercise. The serum TG levels were lower (main effect or overall mean) in the moderately exercised groups ($0.02 < P < 0.05$) and exhaustively exercised group ($P < 0.001$), when compared to the control animals. This is in agreement with previous studies (6–11, 20). The results of this investigation indicate that (i) exercise is effective in lowering serum TG, and (ii) this hypotriglyceridemic response is directly related to the increasing severity of exercise. It appears, that, while this form of exercise has some hypocholesterolemic effect in rats (24), the hypotriglyceridemic response is more pronounced.

The mechanism for this change is not clearly understood. In our previous study (20), we demonstrated that the same exercise program used in this study resulted in an effective increase in thyroid secretion rate (TSR) in both young and mature rats. It is possible that the cause of the decreased plasma TG during exercise may be due to a decreased TG efflux into the plasma. Since

TABLE I. INFLUENCE OF DIFFERENT LEVELS OF PHYSICAL ACTIVITY AND THYROXINE ON SERUM TRIGLYCERIDE CONCENTRATION (mg/100 ml ± SEM)

	Physical activity[a]				
oid status[b] of T_4/day)	No exercise	Standing	Moderate exercise	Exhaustive exercise	Overall mean (A)
	88.9 ± 5.2[c]	69.5 ± 4.2[d]	59.3 ± 3.3[d]	55.7 ± 3.4[e]	68.4 ± 4.0
	47.5 ± 2.8[e]	48.8 ± 4.7	49.1 ± 2.9	42.1 ± 2.6	46.9 ± 3.3[e]
	57.8 ± 3.5[e]	56.9 ± 3.5	51.5 ± 3.2	37.9 ± 2.3[f]	51.0 ± 3.1[e]
	62.9 ± 3.8[e]	48.5 ± 3.0	50.3 ± 3.0	32.9 ± 2.0[d]	48.7 ± 2.9[e]
	43.7 ± 2.9[e]	36.3 ± 2.3	50.7 ± 3.1	33.9 ± 2.1	41.2 ± 2.7[e]
mean (B)	60.2 ± 3.6	58.0 ± 3.5	52.2 ± 3.1[g]	40.5 ± 2.5[e]	

: SD (0.05) for physical activity (B = main effect) = 6.4 mg/100 ml.
: SD (0.05) for thyroid status (A = main effect) = 7.2 mg/100 ml.
: SD (0.05) for AB (interaction) in 14.3 mg/100 ml.
: 0.01, when compared to control group.
: 0.001, when compared to control group.
< P < 0.1, when compared to control group.
0.05, when compared to control group.

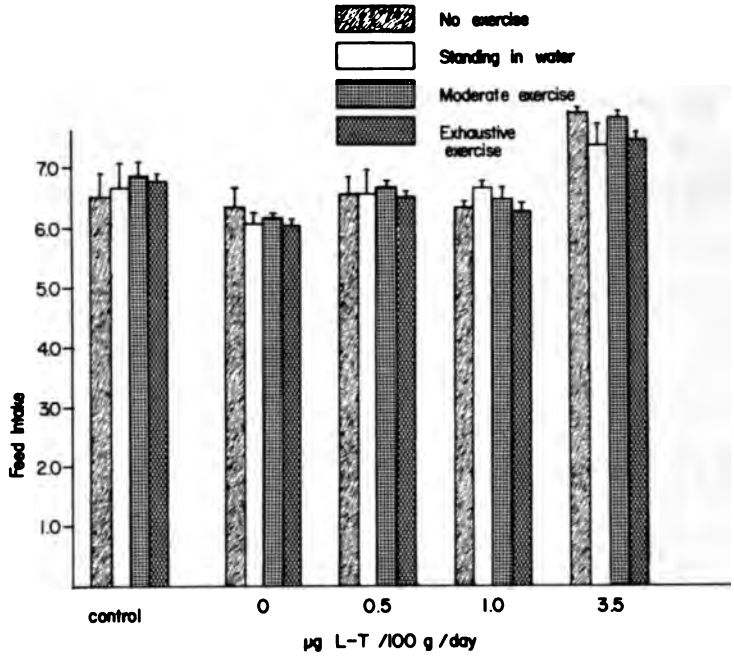

FIG. 1. Feed intake (g/100 g body weight/day) of the four groups of exercised rats (no exercise, stand water, moderate exercise, and exhaustive exercise) that have either intact thyroid (control) or no thyroid (0 L-T/100 g/day) or no thyroid with replacement therapy (0.5, 1.0, or 3.5 μg of L-T/100 g/day). L-T = L-thyr Na salt. Values are expressed as mean ± SEM.

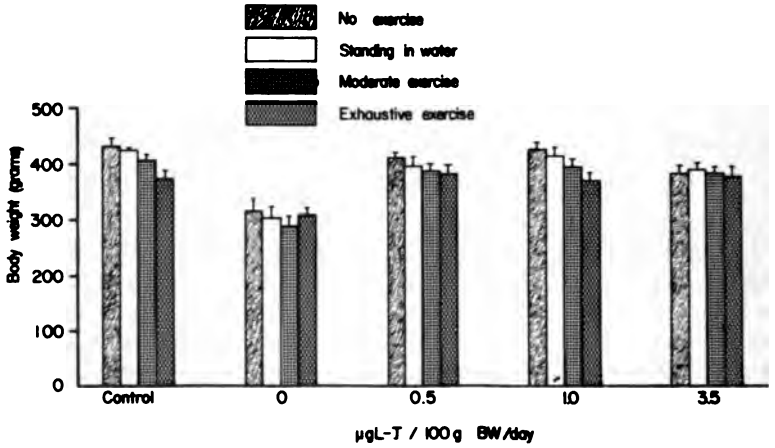

FIG. 2. Body weight (grams) of the four groups of exercised rats that have either thyroid, no thyroid thyroid with daily replacement therapy of L-thyroxine, Na salt. Values are expressed as mean ± SEM.

a large portion of the endogenous plasma TG is derived from the liver, a decreased efflux of TG to plasma could be caused by a decreased secretion of endogenous hepatic TG into the plasma. This might occur as a result of decreased hepatic uptake of plasma FFA for TG synthesis (8, 29). While the mobilization of FFA from adipose tissue is actually increased (30, 31), plasma FF take by the muscles is also increased (33). The increased plasma FFA upta the muscles may be the result of (i) F the preferential fuel for muscles duri elevated metabolic demand of exerc (ii) a change in the distribution of flow during exercise. It is known that

;h the working muscles is in-
?–34), whereas hepatic blood
;iderably reduced during heavy
). This suggests that the uptake
FFA by the liver may be de-
ng exercise, which may result in
formation of TG in the liver.
possible mechanism by which
:ridemia might occur as a result
physical activity may be due to
d influx of TG from the plasma
Nikkilä *et al.* (36) reported that
lipase activity was increased in
o exercise. The increased lipase
eased the FFA available to the
scles for fuel. The increased lip-
ase activity may be the result of
SR due to exercise. Porte *et al.*
d that lipoprotein lipase activity
after thyroxine therapy. In our
dy on rats, we showed that the
d gland is necessary for exercise
ıypocholesterolemia (24). This
ly also suggests that the thyroid
me manner, participates in the
:ridemic effect of elevated physi-

. Male thyroidectomized rats
; levels of daily L-thyroxine in-
: subjected to different amounts
activity to determine the influ-
h factors on serum TG concen-
study indicated that exercise is
owering serum TG and that this
:ridemic response is directly re-
increasing severity of exercise.
animals with intact thyroids had
levels as compared to the thy-
:d rats with differing replace-
of L-T_4 irrespective of the de-
;ical activity. The differing feed
e various groups could not ac-
his hypotriglyceridemic effect.
ıody weight of the thyroidecto-
als, as compared to the control
tact thyroid glands, may be re-
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tudy suggests that the thyroid
me manner, plays an important
ıypotriglyceridemic effect of ex-

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Received July 2, 1976. P.S.E.B.M. 1977, Vol. 154.

S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 377-381 (1977)

arison of the Effects of Secretory Stimulants and Inhibitors on Gastric Mucosal Adenylyl Cyclases of Various Species (37675)

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ntroversial hypothesis about the reg- of gastric acid secretion maintains ion binding to their respective recep- cretory stimulants will then obligato- ivate an adenylyl cyclase of the parie- membrane, thereby initiating an in- ular accumulation of cyclic AMP (1- cording to this concept the increase in llular cyclic AMP is an essential in- iate or "second messenger" for gas- retagogues. There are conflicting re- n the literature concerning the valid- this hypothesis in which the gastric a of a variety of different animal spe- re used (3, 4). In the present study e investigated adenylyl cyclase activi- m the gastric mucosae of four mam- species in response to several secre- imulants (histamine, carbachol and astrin) and inhibitors (prostaglandin retin and epinephrine).

ods. Female Sprague-Dawley rats 0 g), female guinea pigs (400-600 le New Zealand white rabbits (2-4 l male mongrel dogs (15-20 kg) were n these studies. Animals were anes- d and subjected to laparotomy and omy to expose the acid secreting mu- Aucosal scrapings from the corpus of mach were obtained after extensive g from each species. Tissues were ho- ized as 1 g wet weight in 10 ml of *M* sucrose (enzyme grade (-50 m*M* l (pH 7.4, 4°) in a Duall type homog- using five strokes of a Teflon plunger 0 rpm. All homogenates were centri- at 600*g* for 20 min and the pelleted als were then resuspended in 5 ml of mogenizing medium. These particu- eparations were used for this study e had previously found that most of mone and NaF sensitive adenylyl cy- ctivity resided in this fraction of cor- ucosa (5). No unbroken cells were visible in this preparation by light microscopic investigation.

Adenylyl cyclase activities were measured according to Thompson *et al.* (6). Each assay contained 0.2 m*M* ATP, 0.5 μCi ^{32}P-a-ATP (specific activity 4–16 Ci/mmole), 5 m*M* $MgCl_2$, 1 mg phosphocreatine, 5 units creatine phosphokinase, 1 m*M* cyclic AMP, 7.5 m*M* theophylline, and 15 m*M* Tris-Cl (pH 7.4) in a total vol of 0.2 ml. Reactions were initiated by addition of particulate protein (700–850 μg) and the mixture was then incubated for 15 min in the presence or absence of drugs at 30°. The reaction was terminated by addition of 50 μl of 1 *M* acetic acid with vigorous stirring. The ^{32}p-cyclic AMP formed was purified using MnO_2 and Al_2O_3:$PbSO_4$ absorption chromatography (6). Activities were unaffected by solvents for the drugs. Adenylyl cyclase activities are expressed in our results as picomoles of cyclic AMP formed per min per mg protein. Protein was determined by the method of Schacterle and Pollack (7). All reactions were linear with the time of incubation and membrane protein used.

The hormones and drugs tested include histamine acid phosphate (Fisher), carbachol (Regis), pentagastrin (courtesy of B. Mallov, Ayerst), prostaglandin E_1 (courtesy of Dr. John Pike, Upjohn), synthetic secretin (Schwarz/Mann), *d*,1-epinephrine (Regis), tripelennamine (Ciba), chloropheniramine (U.S.V.), and metiamide (Smith, Kline and French), and dimethindine (Ciba).

Results. Figure 1 shows the effects of histamine (1 m*M*), carbachol (1 m*M*), pentagastrin (20 μ*M*), prostaglandin E_1 (0.1 m*M*), secretin (0.1 μ*M*), epinephrine (0.1 m*M*) and NaF (10 m*M*) on the fundic mucosal adenylyl cyclase activities of the 600*g* pellet from rat, dog, rabbit, and guinea pig. The amounts of drug or hormone employed

1977 by the Society for Experimental Biology and Medicine erved.

ISSN 0037-9727

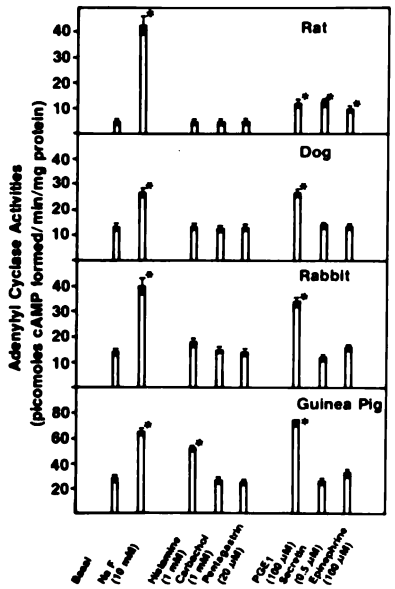

FIG. 1. Effects of secretagogues and Inhibitors of gastric acid secretion on mucosal adenylyl cyclase from various species. * = a significant difference from basal activity ($P < 0.05$).

were previously determined by dose-response curves to be maximally stimulating concentrations were applicable. The basal and hormone stimulated enzyme activities shown were from single representative experiments on one stomach of each species. They are representative of at least five experiments on each species where up to three agents were tested, on each, but not all agents simultaneously as shown here. The adenylyl cyclase activities of each species do not represent the maximum velocity as they were not determined at saturating substrate concentrations. However, in separate experiments, the affinities of the adenylyl cyclase activities of the four species were shown to be similar (Km $\cong$ 0.2 m*M* ATP) and the stimulating agents (histamine, prostaglandin E_1 and NaF) were shown to alter only the maximum velocity and not the Michaelis-Menten constant; therefore, the specific activities as indicated are representative and comparable.

Of the four species tested the guinea pig adenylyl cyclase had the highest basal specific activity. The guinea pig enzyme was also the only system significantly stimulated by histamine, although the rabbit mucosal

ıat produced half maximal inhibition were l in the same range (≅ 0.3 m*M*) but were uch greater than that for the H_2-receptor ıtagonist, metiamide, (≅ 0.01 m*M*) (Fig.).

Discussion. The results of this study indiıte that particulate gastric mucosal adenyl cyclase activity can be stimulated by both hibitors and activators of gastric acid seetion depending upon the species studied. his apparent species variability could conibute considerably to the problem of veriing or disproving the theory that cyclic ucleotides are necessary intermediates in ıe mechanism of action of gastric acid setagogues. Our results constitute the first stematic study of adenylyl cyclase responveness to these agents in several species in hich the enzyme has been localized to a ırticulate cell fraction. Some of our results ınfirm portions of previous studies of oths (2, 9–17) and some conflict with pubhed data (9–11, 13, 16), although the ınflicting reports could involve methodogical shortcomings of previous studies, as ɔ have recently reviewed in detail (4). The lack of histamine stimulation in the rat study was not due to the loss of H_2-receptor since H_2-receptor binding has been identified in a similar particulate fraction from rat gastric mucosa (18).

Except in the guinea pig, the results of these investigations do not support the hypothesis that gastric secretion is initiated by a secretagogue activating particulate adenylyl cyclase on the parietal cell (1–3). Even in the guinea pig mucosa, the other two secretagogues failed to activate this enzyme under our experimental conditions, although it is possible that pentagastrin and the cholinergic agonist could utilize cyclic AMP to mediate their actions on gastric acid secretion by causing the release of histamine (19). This thesis, as far as cyclic AMP is concerned, appears applicable only in the guinea pig stomach. Our studies using antagonists of histamine action indicate that metiamide, an H_2-receptor blocker, is competitive with histamine activation of mucosal adenylyl cyclase and that metiamide has an inhibition constant at least an order of magnitude greater than the three H_1-re-

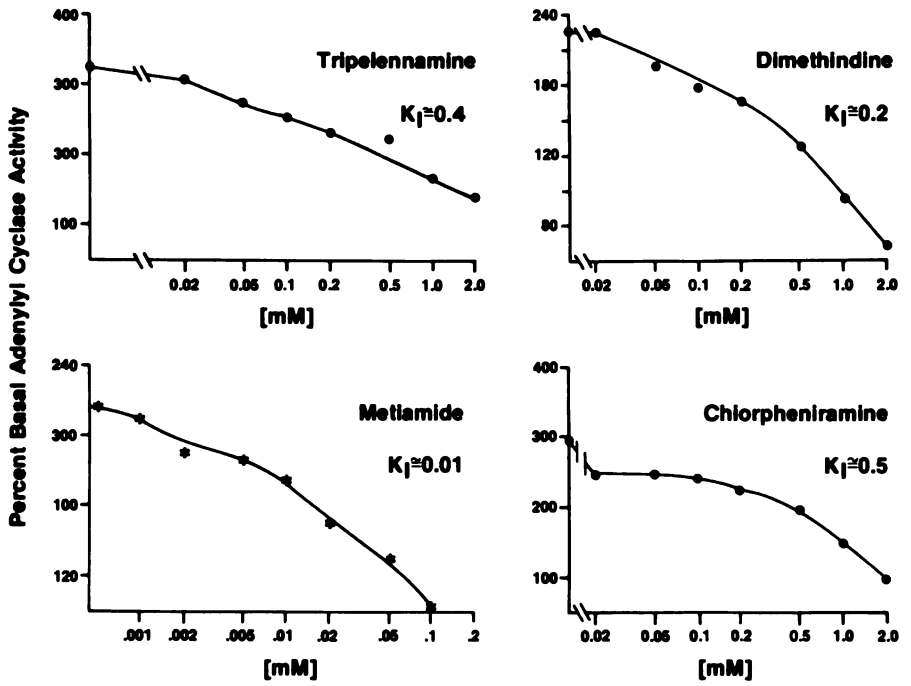

FIG. 3. Inhibition of histamine stimulated gastric mucosal membrane adenylyl cyclase activity by histamine ıtagonists. Enzyme preparation and assay conditions were as indicated in the legend for Fig. 2. All drugs were sted using 100 μM histamine.

ceptor antagonists tested. However, it is not clear from our preliminary studies whether or not the stimulation of adenylyl cyclase by histamine in the guinea pig can occur via a specific histamine H_2-receptor mechanisms, principally because the three H_1-receptor antagonists tested also competed with histamine, albeit with much lower apparent affinities. Other studies have also shown that H_1-receptor antagonists inhibit histamine stimulation of mucosal adenylyl cyclase (14, 17). In addition, all of the antagonists tested inhibited basal and maximally stimulated activity, which suggests a potential nonspecificity of the histamine activation.

Since these crude particulate preparations from each mammalian species contain some parietal cell plasmallemma and the enzyme of only one of the four species tested was activated by histamine, it would appear that cyclic AMP may not be a universal mediator of the actions of gastric acid secretagogues. We cannot determine from these studies whether the guinea pig response represents a true species variance or whether the heterogeneity of the mucosal preparation has contributed to our results. Studies on pure cell preparations from each species will be necessary to differentiate between these possibilities.

The only agent which activated mucosal adenylyl cyclase from each species was prostaglandin E_1, an inhibitor of gastric acid secretion. Since parietal cell membranes are in each adenylyl cyclase preparation, it may even be possible that cyclic AMP is associated with the mechanism of inhibition of gastric acid secretion by prostaglandin E_1. However, each preparation also contains other cell types, any of whose enzymes might be stimulated by the prostaglandin. Recently, an E-type prostaglandin, dibutyryl cyclic AMP and theophylline were shown to stimulate active transport of sodium across gastric epithelial cells (20).

Summary. Adenylyl cyclase activities were measured in mucosal 600*g* particulate preparations from the fundic stomach of rats, dogs, rabbits and guinea pigs. *In vitro* incubation with prostaglandin E_1 and NaF activated the enzyme in all species. Pentagastrin and carbachol were ineffective acti-

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Received June 28, 1976. p.S.E.B.M. 1976, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 382-385 (1977)

Coagulation Studies in C4-Deficient Guinea Pigs[1] (39676)

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The interactions between complement, leukocytes, platelets, and coagluation have been studied rather intensively in recent years (1). The evidence with respect to their direct physiological interaction is conflicting. Rabbits with congenital C6 deficiency have defective intrinsic coagulation (prolonged whole blood clotting times and shortened prothrombin consumption times (2)) and lower responses to injections of complement activators (3) and to slow infusion of thromboplastin (4). In contrast, a human lacking C6 did not have coagulation defects (5). *In vitro* studies of platelet-rich plasma from a patient with C2 deficiency showed less aggregation in response to ADP (6).

Earlier *in vivo* and *in vitro* studies in our laboratories (7, 8) have demonstrated multiple effects on platelet function and coagulation in guinea pigs after intraperitoneal injection of purified cobra venom factor, which is known to deplete C3. Since the link between the coagulation and complement systems requires further definition, we investigated the hemostatic mechanism of congenitally C4-deficient guinea pigs derived from the original NIH-multipurpose strain described by Frank *et al.* (9).

Methods. Sixteen guinea pigs from our randomly-bred closed colony of the NIH-multipurpose, C4-deficient strain were studied. The animals, eight of each sex, were 4-6 months old and weighed over 500 g. The control animals were 16 normal guinea pigs of our randomly-bred Hartley strain matched for age, sex, and weight. Both groups were given the same laboratory rodent food *ad lib*.

For collection of blood samples, the controls and C4-deficients were anesthetized by intraperitoneal injection of sodium pentobarbitol. Samples were taken directly from the posterior vena cava and processed for hematologic, coagulation, fibrinolytic, and complement assays as described previously (8). Coagulation and fibrinolytic assays were performed on plasma from blood anticoagulated with 0.38% trisodium citrate and by the techniques previously cited (8). Blood cell counts and total proteins were measured by standard techniques, and the distribution of serum proteins was determined by electrophoresis on cellulose acetate. Complement activities were titrated by standard cell-intermediate assays (10, 11).

Results. Table I shows the complement activities and plasma proteins in the control and C4-deficient animals. As previously reported (9), the deficient guinea pigs had undetectable C4 activity and significantly lower C1 and C2 activities than the controls. C1 levels were about one-third of normal; C2 levels were about one-half of normal. The combined activity of C3-9 was normal.

The total protein and albumin contents of plasmas from both groups were essentially identical. The proportions and amounts of α_1- and β_2-globulin were significantly lower in the C4-deficients than in the controls. In fact, β_2-globulin was undetectable.

Table II compares the hemograms of the control and C4-deficient guinea pigs. The total leukocyte and platelet counts were significantly greater, and the proportions of segmented, neutrophilic white cells and lymphocytes were significantly different in the C4-deficient group.

The results of hemostatic assays are summarized in Table III. Whole blood clotting times measured in glass and plastic tubes were not significantly longer for the C4-deficient guinea pigs, in contrast to that reported for C6-deficient rabbits (2). The clot

[1] Supported by N.I.H. Research Grant No. HL09902 awarded by the National Heart and Lung *Institute, PHS/DHEW.*

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ISSN 0037-9727

TABLE I. COMPLEMENT ACTIVITIES AND PLASMA PROTEINS IN CONTROL (HARTLEY STRAIN) AND C4-DEFICIENT (NIH STRAIN) GUINEA PIGS.[a]

	Control (n = 16)	C4-Deficient (n = 16)
Complement activity (CH_{50} units/0.5 ml)		
C1	40,000 ± 13,000	13,250 ± 8200[b]
C2	8171 ± 1648	4027 ± 975[b]
C3-C9	632 ± 150	616 ± 136
C4	50,000 – 180,000	Undetectable[b] (<1)
Total protein (g/dl)	4.6 ± 0.3	4.6 ± 0.3
Albumin (% of total)	57.1 ± 3.3	56.9 ± 3.2
Globulins (% of total)		
α_1	5.3 ± 0.8	4.5 ± 0.7[c]
α_2	25.6 ± 1.9	26.8 ± 2.4
β_1	8.4 ± 3.2	9.0 ± 1.0
β_2	1.4 ± 2.4	0[d]
γ	2.2 ± 1.4	2.3 ± 1.0
A:G ratio	1.3 ± 0.2	1.4 ± 0.2

[a] Mean ± SD.
[b] $P < 0.001$.
[c] $P < 0.005$.
[d] $P < 0.025$.

TABLE II. HEMOGRAMS IN CONTROL AND C4-DEFICIENT GUINEA PIGS.[a]

	Control (n = 16)	C4-Deficient (n = 16)
Hematocrit (%)	43.9 ± 3.2	45.3 ± 2.6
Protein (g/dl)	4.6 ± 0.3	4.6 ± 0.3
White blood cells (/mm³)	3850 ± 1068	5160 ± 1500[b]
Platelets (thousands/mm³)	450 ± 65	636 ± 162[c]
Leukocyte differential (%)		
Eosinophils	2.8 ± 2.0	0
Segmenteds	34.2 ± 15.3	23.0 ± 7.7[d]
Lymphocytes	58.0 ± 16.5	72.2 ± 9.3[b]
Monocytes	5.0 ± 2.3	4.8 ± 3.1

[a] Mean ± SD.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P < 0.025$.

retraction of deficient guinea pigs was more active than that of controls, probably because of their higher platelet counts, but clot lysis times and fibrinogen and plasminogen levels were similar in both groups. Of the coagulation screening tests only the prothrombin consumption times with and without phospholipid were significantly different, being shorter in C4-deficients than in controls. Failure of phospholipid addition to correct the prothrombin consumption defect of deficient animals along with the normal Russell's viper venom time indicates normal platelet factor 3 (PF3) activity. Short prothrombin consumption times and normal PF3 activity have also been shown for C6-deficient rabbits (2).

Table IV shows the activities of five clotting factors by sex in the control and C4-deficient animals. There was little variation by sex; some activities appeared to be lower in females, but these differences were not statistically significant. However, when compared to the calculated average plot for the entire age- and sex-matched control group, the C4-deficient group as a whole had significantly lower factor VIII activity. Factor XI activity in this group was significantly higher, especially in females. The lower factor VIII activity of the C4-deficients may be responsible for their slightly longer partial thromboplastin times (Table III).

Discussion. The data indicate that intrinsic coagulation is slower in C4-deficient guinea pigs than in the controls. Whether their moderate C1 and C2 deficiencies contribute to the observed coagulation abnormalities remains to be determined. It may be the combined effect of all three deficiencies that interferes with generation of intrinsic thromboplastin, rather than the C4 deficiency alone.

The defects reported here bear some similarities to those of C6-deficient rabbits, but there are also important differences. The prothrombin consumption defect of C4-de-

ficient guinea pigs is less severe than that of C6-deficient rabbits. Whole blood clotting times were not prolonged in C4-deficient animals, and the lower factor VIII ac observed in this study was not report the C6-deficient rabbits. Although the ulation abnormality in deficient rabbit been attributed to defective platelet- plement interaction (2–4), neither the ent study nor that of Zimmerman *et a* demonstrated abnormal PF3 activity. ther studies of the C4-deficient animal corrected by addition of purified C4 a C142 and, (ii) whether C4-deficient an reconstituted by bone marrow transpl tion (11) still have the clotting defects

The significance of hemostatic abnor ties observed in various congenitally plement-deficient animal species is op question. As Brown (1) has pointed complement-deficient humans do no pear to manifest these defects on *in* assays. Conversely, patients with inhe coagulation disorders do not appear to significant complement abnormalities although this possibility has not been e sively studied. The clinical syndromes monly associated with inherited con ment deficiencies (infections, renal dis lupuslike disorders) are not usually see patients with inherited coagulopat However, the failure to demonstrate relationships in the few patients studied far is not conclusive. Considering the variability in normal ranges observed fo coagulation and complement system may be impossible to demonstrate su

TABLE III. HEMOSTATIC VALUES IN CONTROL AND C4-DEFICIENT GUINEA PIGS.[a]

	Control (n = 16)	C4-Deficient (n = 16)
Whole blood clotting time (min, n = 6)		
Glass	2.9 ± 0.4	3.0 ± 0.0
Plastic	4.7 ± 1.1	4.1 ± 0.7
Clot retraction (% at 2 hr)	78.9 ± 6.2	83.6 ± 5.6[b]
Clot lysis time (hr)	17.2 ± 8.6	20.0 ± 12.3
Fibrinogen (mg/dl)	240.0 ± 44	229.0 ± 26
Plasminogen (CTA U/ml)	2.8 ± 0.7	2.5 ± 0.8
Activated partial thromboplastin time (sec)	23.1 ± 2.3	25.1 ± 3.6
One-stage prothrombin time (sec)	35.3 ± 2.3	35.6 ± 3.4
Russell's viper venom time (sec)	10.2 ± 1.1	9.6 ± 0.8
Prothrombin consumption time (sec)	96.5 ± 28.9	61.8 ± 19.8[c]
Prothrombin consumption time with phospholipid (sec, n = 6)	79.8 ± 15.0	54.3 ± 7.4[c]

[a] Mean ± SD.
[b] $P < 0.05$.
[c] $P < 0.001$.

TABLE IV. COAGULATION ACTIVITIES (%) IN CONTROL AND C4-DEFICIENT MALE AND FEMALE GUINE PIGS.[a]

Group and factor	Males (n = 8)	Females (n = 8)	Total (n = 16)
Controls[b]			
VII	116 ± 37	90 ± 25	103 ± 34
VIII	102 ± 42	112 ± 54	107 ± 47
IX	125 ± 52	97 ± 56	111 ± 54
X	118 ± 43	94 ± 26	106 ± 34
XI	104 ± 40	92 ± 25	98 ± 33
C4-Deficients[b]			
VII	103 ± 28	76 ± 21	90 ± 28
VIII	62 ± 27	58 ± 20	60 ± 23[c]
IX	121 ± 69	117 ± 44	119 ± 57
X	89 ± 25	92 ± 16	91 ± 21
XI	109 ± 36	179 ± 63	144 ± 50[c]

[a] Mean ± SD.
[b] For each factor the mean clotting time at each assay dilution for the entire control group was plotted and plot was assigned a value of 100%. The plot for individual control and deficient animals was compared to t mean plot and the results were averaged for each group.
[c] *Control vs. deficient* values: $P < 0.005$.

ifferences without studying larger numbers f patients and/or more carefully selecting nd standardizing the control population sed for comparison.

Summary. C4-deficient guinea pigs (NIH-nultipurpose strain) were found to have ower intrinsic coagulation activity than age- nd sex-matched control guinea pigs (Har- ley strain).

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Received September 21, 1976. P.S.E.B.M. 1977, Volume 154.

Mechanisms by which Retrobulbar Fibrobl of Cyclic Nucle

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Accumulations of glycosaminoglycans (GAG) and aggregations of lymphocytes in orbital tissues are characteristic features of active Graves' eye disease (1, 2). Cultures of fibroblasts derived from human retrobulbar connective tissue produce a specific GAG, hyaluronic acid (HA), whose synthetic activity is stimulated by lymphocytes and lymphocyte products (3–5). The increased synthesis of HA by cultured cells from the human orbit may provide an analogy to Graves' ophthalmopathy. It is of interest, therefore, to investigate the intracellular processes by which stimulation takes place.

Cyclic 3′,5′-adenosine monophosphate (cAMP) is a mediator of many intracellular changes including the stimulation of thyroid cells by thyrotropin (6) and the enhanced HA synthesis by synovial fibroblasts (7). The studies reported in this communication demonstrate in retrobulbar fibroblasts enhanced synthesis of HA in response to dibutyryl cyclic AMP (DbcAMP) and to theophylline and a rise in intracellular cAMP after exposure to lymphocytes. These findings support the concept that cAMP mediates the lymphocyte stimulation of HA synthesis in the cultured cells. Hydrocortisone, which inhibits the lymphocyte stimulation of fibroblasts (4, 5), appears to do so by interfering with the intracellular cAMP response to the stimulus.

Materials and methods. Cultures of fibroblasts derived from the normal human retrobulbar connective tissue of two patients were initiated and perpetuated as described in an earlier publication (4). For experi-

[1] This study was carried out under the auspices of United States Public Health Service Grant No. AM 17538. Presented in part at the Central Society for Clinical Research in Chicago, November 8, 1975, and *appearing in abstract* form in Clin. Res. 23, 517A *(1975).*

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residual glucose and of GAG which ressed as uronic acid (3). The fibroeets were washed three times with l saline and analyzed for protein (4). riate blank cultures without fibroere included to ascertain initial glul GAG values in media.

:ellular cAMP was extracted from ihed fibroblast sheet in the flasks ó trichloroacetic acid (TCA). Ethyl turated with water was then used to the TCA from the aqueous phase ng the cAMP; after evaporation, IP residues were taken up in 0.05 *M* phosphate buffer, pH 6.2, and the rations measured by radioimmuusing kits purchased from Schwarz/ Recovery of cAMP was monitored inting [^{3}H]adenosine, previously o the TCA, in the final solutions, as :d by Sato *et al.* (8).

iature of the GAG after extraction edia was determined as described (3). Intrinsic viscosities of HA in cted media were determined by rescometer flow rates observed before :r the addition of hyaluronidase, a described by Castor and Prince (9). iults are relatively specific for the / of HA and were used to calculate ar weights of the HA produced by ıltures.

tical comparisons were by Student's

ts. Evidence for cyclic AMP as a r in the stimulation of HA synthesis. Addition of DbcAMP to the cultures of retrobulbar fibroblasts produced dose-dependent increases in the media GAG (Table I). Fibroblast protein was unchanged except at the highest doses of DbcAMP where small increases or decreases in the mean cellular protein were observed. Glucose utilization by the fibroblasts was stimulated less than GAG by DbcAMP; not until DbcAMP concentrations reached 2×10^{-3} *M*, or higher, was the glucose utilization increased by 20% or more. The increases in glucose utilization following additions of DbcAMP were also less than those observed after the additions of lymphocytes which gave comparable stimulation in GAG synthesis (Table II).

Theophylline, at 10^{-4} *M* concentration, induced a modest increase in media GAG but potentiated the GAG responses to lymphocytes (Table II). In separate experiments, additions of dimethyl sulfoxide in quantities that contaminate the lymphocyte preparation had no effect on any measurement made on the cultured fibroblasts.

Two hours after the addition of frozen-thawed lymphocytes, the concentration of intracellular cAMP in the fibroblasts was significantly increased (Table III). Shorter periods of incubation gave inconsistent stimulation of cAMP.

Inhibitory action of hydrocortisone. Hydrocortisone reduced the concentration of the cyclic nucleotide within the cultured cells to an insignificant degree (Table III). But this steroid significantly inhibited the

TABLE I. EFFECTS OF DIBUTYRYL CYCLIC AMP ON HUMAN RETROBULBAR FIBROBLASTS.[a]

P conıtion $^{-3}$ *M*)	No. of flasks	GAG in media (nmole of uronic acid/mg of fibroblast protein)[b]	(E/C)[c]	Glucose utilized (μmole/mg of fibroblast protein)[b]	(E/C)[c]
		Experiment No. 1, fibroblasts from patient A			
)	(5)	122 ± 3		11.9 ± 0.1	
2	(5)	155 ± 4	1.28	11.6 ± 0.3	0.97
5	(5)	198 ± 6	1.63	11.9 ± 0.3	1.00
)	(5)	275 ± 7	2.26	12.2 ± 0.3	1.02
5	(5)	422 ± 9	3.48	12.0 ± 0.1	1.01
		Experiment No. 2, fibroblasts from patient B			
)	(4)	54 ± 2		11.6 ± 0.8	
)	(3)	489 ± 20	9.0	12.9 ± 0.7	1.11
)	(3)	912 ± 24	16.8	17.3 ± 0.4	1.49
)	(4)	1330 ± 69	24.5	18.0 ± 0.8	1.55

ia collected over Days 2-4 of culture; fibroblast protein determined on Day 4.
n ± SEM.
:rimental group mean ÷ control group mean.

TABLE II. THEOPHYLLINE POTENTIATION OF LYMPHOCYTE STIMULATION OF GAG PRODUCTION BY RETROBULBAR FIBROBLASTS.

Concentrations of additives	No. of flasks	GAG in media[a] (nmole of uronic acid/mg of fibroblast protein)[b]	GAG in media[a] (increments above basal concentrations)
None	(5)	44 ± 2	
Theophylline, 10^{-4} *M*	(3)	65 ± 6	21
Lymphocytes, 10^5/ml	(3)	76 ± 4	32
Lymphocytes, 5 × 10^5/ml	(3)	145 ± 4	101
Lymphocytes, 10^5/ml, plus theophylline, 10^{-4} *M*	(3)	186 ± 3	142
Lymphocytes, 5 × 10^5/ml, plus theophylline, 10^{-4} *M*	(3)	298 ± 5	254

[a] Media collected over Days 2–4 of culture.
[b] Mean ± SEM.

TABLE III. EFFECTS OF LYMPHOCYTES AND HYDROCORTISONE ON INTRACELLULAR cAMP IN RETROBULBAR FIBROBLASTS

Additions to media[a]	No. of flasks	Intracellular cAMP (pmole/mg of fibroblast protein, mean ± SEM)
None	(4)	33.5 ± 3.9
Lymphocytes,[b] 5 × 10^5	(4)	70.0 ± 5.8[c]
Hydrocortisone, 1 μg/ml	(4)	29.0 ± 2.4
Lymphocytes,[b] 5 × 10^5, plus hydrocortisone, 1 μg/ml	(4)	37.6 ± 4.0

[a] Incubation for 2 hr
[b] Lymphocytes, frozen-thawed, contained less than 25% of cAMP found in unstimulated fibroblasts; much of this added cAMP would have been discarded with removal of the media.
[c] Differed from each group, $P < 0.01$.

lymphocyte stimulation of intracellular cAMP within the fibroblasts (Table III). However, hydrocortisone did not reduce the synthesis of GAG induced by DbcAMP (2 × 10^{-3} *M*): media GAG following fibroblast exposure to DbcAMP alone was, in nanomoles of uronic acid per milligram of fibroblast protein, 445 ± 28 (over six times the control level) and after exposure to both DbcAMP and hydrocortisone was 565 ± 25. Indeed, there was a mild potentiation of the DbcAMP effect when hydrocortisone was added.

Differences in fibroblast synthesis of GAG in response to DbcAMP and lymphocytes. By paper chromatography and by differential solubility in Na_2SO_4-CPC, the GAG produced by the DbcAMP-stimulated fibroblasts appeared as HA (Table IV). When the steps of extraction of culture media are taken into account, the reproducibility of measurements of uronic acid are within 10%, and hexosamine within 15%. These variabilities in results deviate the hexosamine/hexuronic ratios in Table IV from the theoretical value of 1 for HA, but, within the error of assays, the ratios are consistent with the concept of HA as the only GAG present in the media. The absence of galactosamine in the GAG weighs strongly against appreciable quantities of chondroitin sulfates and dermatan sulfate as constituents of the GAG, an observation which points to HA as the major, if not the only, component.

Measurements of intrinsic viscosity on unextracted media indicated differences in the molecular sizes of the HA synthesized after exposures to lymphocytes and to DbcAMP (Table IV). The intrinsic viscosity of the HA produced by fibroblasts after lymphocyte stimulation was lower than basal values while that of the HA synthesized after additions of DbcAMP was higher; the molecular weights of HA, calculated from the respective intrinsic viscosities, were accordingly different: 2 × 10^5 and 5 × 10^6 daltons. In separate experiments, incubation of authentic HA in media with lymphocytes or DbcAMP, added in concentrations similar to those used in the above experiments, resulted in no change in the intrinsic viscosity after 3 days.

Discussion. In this communication, changes in concentrations of GAG (shown to be HA) in the media of fibroblast cultures was attributed to alterations in synthesis of the polysaccharide. There is no evidence

IV. NATURE OF GAG IN MEDIA AFTER RETROBULBAR FIBROBLAST STIMULATION BY LYMPHOCYTES AND DIBUTYRYL CYCLIC AMP.

	Experiment No. 1 Additives to Cultures		Experiment No. 2 Additives to Cultures	
	None	Lymphocytes, 5×10^6/ml	None	DbcAMP, 2×10^{-3} *M*
f extracted GAG soluble in 0.2 *M* PC solution[a, b]	0.73	0.80	0.82	1.00
AG on paper chromatography[b]				
nium-methanol-formate solvent[c]	HA	HA	HA	HA
0.1 *M* solvent [c]	HA	HA	HA	HA
ıe/hexuronic acid ratio[b]	0.77	0.90	0.87	1.06
hexosamine on paper chromatogra-	Only glucosamine	Only glucosamine	Only glucosamine	Only glucosamine
iscosity[b]				
gram of HA	11.2	4.3	13.6	61.1
molecular weight of HA (millions)	0.6	0.2	0.8	5.0

ıluble in this solution.
recorded in Ref. (3) and (9).
ority, but not the total, stainable GAG was in the HA fractions in all cases.

is degraded by the fibroblasts, and cellular content of GAG was found v compared to that in media (10); , it is unlikely that the observed in media GAG were derived from ed degradation or from release racellular stores. Further, in un- l studies, it was found that osamine was incorporated into me- n patterns consistent with the con- experimental manipulations affect thesis rates.

cAMP mediates the lymphocyte on of HA synthesis in retrobulbar ts is indicated by the observations DbcAMP produced the same type esponse; (b) theophylline, a phos- erase inhibitor, potentiated the yte action; and (c) exposure of ions to lymphocytes induced an in- ı the cAMP concentration in the ts within 2 hr. Although a previous on from this laboratory reported MP added to retrobulbar fibroblast produced no effect (5), it seems at the cAMP then used was either legraded or did not penetrate cells as the dibutyryl derivative of the le.

ver, lymphocytes affected two re- in the cultured cells that differed ose observed after additions of P. Relative to the effects on HA , DbcAMP consistently increased ıtilization less than did the lympho- A was the only type of GAG found e media after the addition of either stimulating agent, but the intrinsic viscosity (and, therefore, the molecular weight) of HA decreased as a consequence of lymphocyte action, and increased as a result of the presence of DbcAMP. It is likely that the stimulation produced by lymphocytes resulted in changes in glucose metabolism separate from those in the adenyl cyclase system; the former effects may play a role in modifying the polymer size of HA.

Hydrocortisone was previously reported to inhibit the lymphocyte stimulation of HA synthesis in the fibroblast cultures (4, 5). Hydrocortisone also markedly reduced the intracellular cAMP response to lymphocytes. Thus, the inhibitory effects of the steroid hormone occur at an early stage in the events consequent to the interaction of lymphocytes and fibroblasts. Possibly hydrocortisone prevents binding of the active factor within lymphocytes to a fibroblast receptor.

In contrast, hydrocortisone did not interfere with but rather potentiated the DbcAMP effects on HA synthesis in the cultures. The potentiation was possibly related to unmeasured intracellular actions of hydrocortisone on retrobulbar fibroblast metabolism.

Summary. It was shown that lymphocytes and lymphocyte products stimulated hyaluronic acid (HA) synthesis in cultures of human retrobulbar fibroblasts. This stimulation was simulated by dibutyryl cyclic AMP (DbcAMP), 2×10^{-4}–10^{-2} *M*, and potentiated by theophylline, 10^{-4} *M*. Lymphocytes induced a significant increase in the

cAMP concentration within the fibroblasts. These data support the concept that the lymphocyte stimulation of HA synthesis is mediated by cAMP.

However, lymphocytes induced greater glucose utilization by the cultures than DbcAMP. Also, the intrinsic viscosity of the HA was decreased by lymphocytes but increased by DbcAMP. Lymphocytes probably produce more effects in the fibroblasts than those mediated by cAMP.

Hydrocortisone inhibited both the cAMP and the HA responses to lymphocytes but potentiated the stimulation of HA synthesis by DbcAMP. Hydrocortisone thus appears to interfere with the earliest effects of lymphocytes, possibly inhibiting the binding of the active factor in lymphocytes to retrobulbar fibroblasts.

Dr. C. W. Castor provided valuable advice in the preparation of this manuscript. Expert technical assistance provided by Piyush Kothary, Deborah Weiker and Howard Kirchick was greatly appreciated. The author is also pleased to acknowledge the expert editorial assistance of Cornelia Osborn.

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Received July 6, 1976, P.S.E.B.M. 1977, Vol. 154.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 391-396 (1977)

Levodopa on Arterial Blood Pressure in Unanesthetized and in Anesthetized Rats (39678)

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e of levodopa in the treatment of ism has led to an increased inter- physiological and pharmacological this drug, as well as to an aware- cardiovascular and, to a smaller ther derangements occur during therapy. For this reason the ef- vodopa on the cardiovascular sys- been studied extensively. The re- ined are somewhat contradictory. ple, it has been reported that levo- eases (1–3) or increases (4–9) the lood pressure. The difference in ight be due to the choice of the ecies or to the difference in admin- ses of levodopa (16, 32). Anes- l surgical procedures may also be variability of the obtained results. n shown that anesthesia and sur- oundly change cardiac output, ar- od pressure, and heart rate in ex- l animals (10).

ain purpose of this work was to acute effects of levodopa on the lood pressure in unanesthetized thetized rats after chronic aortic on. The mean arterial blood pres- recorded before and after single ation of levodopa.

ls and methods. Forty-two adult rague–Dawley rats weighing 198 g were used in the experiments. nal was housed in a separate cage given food and water *ad lib.* The ach animal was cannulated under (Fluothane, kindly supplied by boratories) anesthesia with a fine ne tubing (PE 10) via the left ca- y 3–4 wk prior to the actual blood measurement (11, 12). After full from surgery and anesthesia, the ontinued to grow following the curve of growth for this species ddition to the chronic aortic can- ats were also carriers of a chronic right ventricular heart cannula (PE 10) permitting the iv administration of levodopa (12).

During blood pressure recording, each animal was placed in a plastic 4″ × 10″ × 4″ box. A 1/4-in. wide opening in the middle of the cover of the box permitted connection of the implanted cannula to the transducer and free movement within the box during voluntary locomotion of the rat. The implanted cannula of the animal was connected by a needle adapter to a pressure transducer (P23 De, Statham) and polygraph (Beckman). The pressure was recorded after the animal reached a complete resting state, 5–15 min after placement in the box. Each measurement after levodopa administration lasted one hour.

Levodopa (Hoffman La Roche) was dissolved in warm saline and administered intra-arterially through the chronically implanted aortic cannula. In six rats the levodopa was administered iv through the right ventricular heart cannula. The volume of administered fluid (saline as a solvent) never exceeded 0.5 ml. The arterial blood pressure measurements were made between 9 and 12 AM. Besides the mean arterial blood pressure, body weight, body temperature, and hematocrit ratio were monitored. Nembutal (sodium pentobarbital) was given ip, 40 mg/kg. Halothane was mixed with oxygen and introduced through a mask placed over the nose and the mouth of the rat. The flow rate of halothane and oxygen was maintained at a constant level. Depth of anesthesia was judged by breathing rate and corneal blink reflex. However, variations in the respiration rate and the duration of anesthesia probably have effects on the depth of anesthesia. Levodopa was administered 10 min after the animals reached the desired levels of anesthesia.

Statistical treatment consisted of single factor analysis of variance on repeated

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ISSN 0037-97

measures. Significance at the $P = 0.05$ level was determined from the least significant difference procedure for pairs of means.

Animal groups. All of the rats were randomly separated into six groups, each group consisting of six animals. The groups were:

Unanesthetized rats. 1. 10 mg/kg levodopa, i.a. injection. 2. 20 mg/kg levodopa, i.a. injection. 3. 10 mg/kg levodopa, iv injection.

Anesthetized rats. 4. 10 mg/kg levodopa, i.a. injection and nembutal anesthesia. 5. 20 mg/kg levodopa, i.a. injection and nembutal anesthesia. 6. 10 mg/kg levodopa, i.a. injection and halothane anesthesia.

Results. Mean arterial blood pressure after a single intravascular injection of 10 mg/kg of levodopa. Administration of nembutal or of halothane decreased the arterial blood pressure from 120 mmHg to 80–85 mm Hg. After administration of levodopa (10 mg/kg, ia), the arterial blood pressure increased in unanesthetized and in anesthetized rats (Fig. 1). The rise in arterial blood pressure reached a peak after 2–5 min in all groups. The increase of the arterial blood pressure in unanesthetized rats 2–5 min after i.a. levodopa administration was between 35 and 50 mmHg (Fig. 2). Fifteen minutes after levodopa administration, the arterial blood pressure of the unanesthetized rats was close to the control preadministration level, where it stayed during the next 45 min. The return to control values was somewhat slower in anesthetized rats. Intravenous administration of levodopa caused a larger increase of the arterial blood pressure in unanesthetized rats than the intra-arterial injection of the same dose.

In the nembutal group the increase of the arterial blood pressure after 2–5 min was 90 mmHg, much greater ($P < 0.05$) than in other groups (Fig. 2 and Table I). After levodopa administration, the increase of the mean arterial blood pressure was smaller with halothane than with nembutal anesthesia (Figs. 1 and 2).

The composite analysis of the observed changes of the arterial blood pressure induced by 10 or 20 mg/kg of levodopa is given in Table I.

Mean arterial blood pressure after a single intra-arterial injection of levodopa (20 mg/

TABLE 1. COMPOSITE STATISTICAL EVALUATIONS* OF MEAN ARTERIAL BLOOD PRESSURE CHANGES 2-5 MIN AND 15 MIN AFTER LEVODOPA ADMINISTRATION TO UNANESTHETIZED AND ANESTHETIZED RATS.

Animal Group	Time, min					
Unanesthetized Levodopa, 10 mg/kg i.v.	2-5 15	NS NS				
Unanesthetized Levodopa, 20 mg/kg i.a.	2-5 15	<.05 NS	NS NS			
Halothane Levodopa, 10 mg/kg i.a.	2-5 15	NS NS	<.05 NS	<.05 NS		
Nembutal Levodopa, 10 mg/kg i.a.	2-5 15	<.05 <.05	<.05 <.05	<.05 <.05	<.05 <.05	
Nembutal Levodopa, 20 mg/kg i.a.	2-5 15	<.05 <.05	<.05 <.05	<.05 <.05	<.05 <.05	NS <.05
Animal Group	Time, min	Unanesthetized Levodopa, 10 mg/kg i.a.	Unanesthetized Levodopa, 10 mg/kg i.v.	Unanesthetized Levodopa, 20 mg/kg i.a.	Halothane Levodopa, 10 mg/kg i.a.	Nembutal Levodopa, 10 mg/kg i.a.

* Single factor analysis of variance on repeated measures. Significnce at the $P = 0.05$ level was determined from the least significant difference procedure for pairs of means. All P values $<.05$ are accepted as significant.

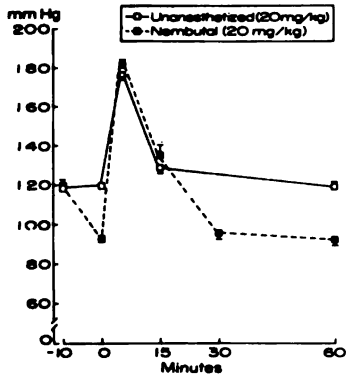

FIG. 3. Mean arterial blood pressure (±SE) of adult female rats after intraarterial levodopa (20 mg/kg) administration.

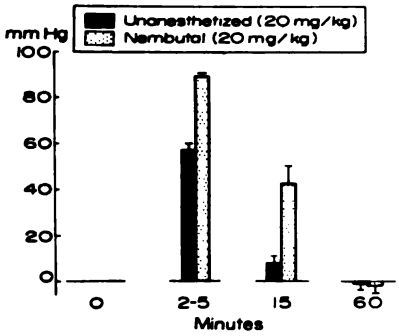

FIG. 4. Increases of the mean arterial blood pressure (±SE) of adult female rats after intra-arterial levodopa (20 mg/kg) administration.

No changes in body weight, body temperature and hematocrit ratio were observed after administration of levodopa.

Discussion. Levodopa therapy causes some derangements of the cardiovascular system (13). Long-term administration of oral levodopa to patients with Parkinson's disease induces orthostatic hypotension in about 30% of the cases (14, 15). The cardiovascular effects of levodopa are associated mainly with dopamine. It has been reported that dopamine stimulates myocardial β-adrenergic receptors (6) leading to increased cardiac contractile forces (16-20) and to an increased cardiac output (21). These effects were used beneficially in the treatment of cardiogenic and noncardiogenic shock (17, 20, 22-24). Dopamine causes hypertension largely by direct interaction with α-adrenergic receptors in vascular smooth muscle replacing (25) or releasing the stores of norepinephrine from sym-

mean arterial blood pressure after
erial administration of levodopa.
rease was greater and appeared to
er when the levodopa dose was dou-
r the dose of 10 mg/kg of levodopa,
ease of the arterial blood pressure
ater if levodopa was administered
ously instead of intra-arterially. We
that this might be associated with
ct action of levodopa on myocar-

esults on unanesthetized animals
npared to the values obtained in the
during nembutal or during halo-
esthesia. The later part of this work
e because it is known that anesthe-
profoundly cardiovascular parame-
). Furthermore, it was reported that
ie causes myocardial depression and
pamine either decreases or leaves
ed the total peripheral resistance in
ie anesthetized animals (24). The
ie content of the brain is increased
esthesia, as well as dopamine syn-
id turnover (37).
r work on nembutal and on halo-
nesthetized rats, we found an in-
man arterial blood pressure after
tion (ia) of levodopa. The increase
ch smaller in halothane anesthesia.
ect might be associated with the de-
action of this anesthetic on the
id its profound effects on the CNS.
ation of the hypertensive response
d to be longer in nembutal anesthe-
imals that received a larger dose of
a. It is interesting to note that the
lue of the mean arterial blood pres-
not much different if 10 or 20 mg/
odopa was given to nembutal anes-
rats.
ary. The arterial blood pressure re-
in rats after intravascular levodopa
tration were measured for the first
h direct methods in unanesthetized,
ined animals after full recovery
gical stress due to the cannulation.
served results were compared with
ollected from anesthetized animals
tal or halothane). After levodopa
tration, the mean arterial blood
of all rats was increased, reaching
value 2–5 min later. The animals in
ie anesthesia had a small rise of the

mean arterial blood pressure. Doubling the dose of levodopa increased further the hypertensive responses in unanesthetized animals but not in nembutal anesthesia. Percentage-wise, levodopa caused a much greater increase of the mean arterial blood pressure in nembutal groups than in unanesthetized animals. However, the peak values were the same, around 170–180 mmHg, in all nembutal anesthetized animals as in unanesthetized animals that received a double dose of levodopa (20 mg/kg).

This research was supported by the Office of Naval Research Contract No. N00014-76-C-0926 with funds provided by the Naval Medical Research and Development Command.

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Received May 24, 1976. P.S.E.B.M. 1977, Vol. 154

Y FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 397–400 (1977)

ɔe upon the Transport Kinetics of *p*-Aminohippurate at 37°C in Rabbit Kidney Slices (39679)

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stimulatory effect on the -aminohippurate transport (1) and *in vivo* (2). This ıterpreted as a direct effect ɛlated to the transport sys- .ct, Cross and Taggart (1) ıe stimulation of *p*-amino- ulation seen in adult kid- ıt acetate is one of the rate- ɔomponents involved in the n. On the other hand, (4) suggested that acetate ıohippurate uptake by its glycine to form acetylgly- ɔtions to relieve a normal etitive inhibition by long ɔs. The suggested relative red by acetate (1–4) as a t was challenged by Koishi that acetate inhibited, as ed, *p*-aminohippurate up- yruvate, succinate, and α- ıantitatively mimicked the y acetate. The controversy ole of acetate in the active ɔrganic acids by the kidney ıerform comparable studies ppurate as a model weak ɔrder to elucidate the spe- acetate.

nethods. Adult New Zea- ghing from 2.5 to 4.0 kg is investigation. After the ıcrificed by injecting air ular vein, the kidneys were oved and placed in an ice- ı (10 mM) Cross-Taggart of acetate. Renal cortical ıtely 0.3–0.5 mm thick and 0 mg, were cut with a Sta- slicer and then were ran- ıto two groups. One group ɔ a modified low-Na Cross- bation medium containing , whereas the other was transferred to a similar preincubation medium devoid of acetate. Preincubation was done at 0° for 30 min. The groups of slices were incubated in their appropriate high-Na (100 m*M*) modified Cross-Taggart medium containing *p*-aminohippurate, in either the presence or absence of 10 m*M* acetate, respectively. The compositions of the 10 and 100 m*M* Na media have previously been described (6). In the nonacetate series, sodium acetate was replaced by an equimolar amount of NaCl. All preincubations and incubations were performed under 100% O_2. The incubations were done in a Dubnoff metabolic shaker at either 25 or 37° for 45 min.

Three different types of experiments were performed. In the first series of experiments, the effect of acetate on *p*-aminohippurate accumulation and oxygen consumption at both 25 and 37° was examined. Groups of slices were preincubated and incubated in either the presence or absence of acetate. In the accumulation experiments, the slices were blotted and weighed at the end of the incubations; the calculations were based upon this wet weight. In the oxygen consumption experiments, the slices were blotted and weighed prior to incubation. Oxygen uptake was represented on the basis of this initial wet weight, because some of cell components were thought to disperse into the medium and consume oxygen, though these would be excluded from the final wet weight of the slices.

The second series of experiments was a kinetic study similar to that described by Gerencser *et al.* (6). At the end of incubation, the slices were immediately removed, blotted, and weighed. The slices and the incubation media were then analyzed for *p*-aminohippurate.

In a third series of experiments, we examined the effect of acetate on the energy charge potential (7), focusing on the adeno-

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ISSN 0037-9727

sine 5′-triphosphate concentrations in the slices. Following preincubation, the slices were blotted and weighed, after which they were incubated in either the presence or absence of 10 m*M* acetate at 37°. After incubation, slices were frozen in liquid nitrogen until the analyses for nucleotides were done.

Calculations were based on tissue wet weight. The concentration of *p*-aminohippurate in the slices and in the medium was determined by the methods of Smith *et al.* (8). The oxygen consumption of the slices was measured using a Warburg manometric respirometer as described by Cross and Taggart (1). Adenosine triphosphate analyses were performed as described by Lamprecht and Trautschold (9). Adenosine 5′-diphosphate and adenosine 5′-monophosphate were determined as described by Adam (10). Energy charge potential was calculated as defined by Atkinson (7).

All data are reported as means ± SE. Differences between means were analyzed statistically using the Student's *t*-test. Where appropriate, paired comparisons were made. The 0.05 level of probability was used as the criterion of significance.

Results. The first series of experiments examined the effects of acetate on the accumulation of *p*-aminohippurate at both 25 and 37° (Table I). The slice to medium ratio (S/M) for *p*-aminohippurate increased significantly, by approximately 50%, at both temperatures. It is also of interest to note that there was no significant difference in the corresponding S/M values between the two temperatures in either the presence or absence of acetate, indicating, in agreement with the results of Cross and Taggart (1), that the uptake of *p*-aminohippurate reaches a maximum at 25°.

In contrast, the next group of experiments showed that acetate induced a significant increase in the oxygen consumption at 25° but not at 37° (Table II). Moreover, the oxygen consumption was significantly higher at 37 than at 25° in both the presence and absence of acetate ($P < 0.05$).

The rate of *p*-aminohippurate uptake at 37° as a function of *p*-aminohippurate concentration in the incubation medium is *shown in Fig.* 1. The rate of uptake in-

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rease in V_{max} (the intercept with the te) but no change in K_m (the slope of e) by acetate. The K_m of slices in the f acetate and acetate-free media was : 0.08 and 0.27 ± 0.05 m*M*, respec- in six paired experiments (not signifi- different from one another). The f the same slices in the 10 m*M* acetate etate-free media was 2.99 ± 0.52 and : 0.20 mM/45 min·kg, respectively ;). The mean V_{max} of slices bathed in m*M* acetate medium was significantly than the mean V_{max} of slices bathed acetate-free medium ($P < 0.05$ for *t*-test). It is evident that acetate sig- tly increased V_{max} but had no effect K_m.

next series of experiments examined ect of acetate on energy charge poten- 37°. The tissue concentrations of var- ucleotides and the calculated energy potential at 37°C are summarized in III. Acetate significantly decreased evel of adenosine triphosphate, is it significantly ($P < 0.05$) in- l that of adenosine diphosphate. 'er, acetate had no significant effect energy charge potential.

ussion. The results obtained in the t work indicate that acetate increases take of *p*-aminohippurate by about t both 25 and 37° (Table I), whereas eases the oxygen consumption at 25 t at 37° (Table II). Such a dissociation ate on the transport and metabolism suggests that the stimulatory effect of : on organic acid transport is not me- by the stimulation of metabolic en- 1). However, a possibility that the to increase oxygen consumption by : at 37° might be due to the limitation ision of oxygen from the medium into the the slice should be considered. Accord- ing to the Warburg criteria (11),

$$b' = \left(\frac{8D \cdot Uo}{\alpha}\right)$$

where b' = the limiting thickness of the tissue slice into which oxygen can diffuse from both sides of the slice; D = Krogh diffusion coefficient of oxygen in water (1.7×10^{-5} cm^2·min^{-1}·atm^{-1} at 37° (11); Uo = oxygen pressure in incubation medium (1 atm with oxygenation); and α = oxygen consumption. When $b' = 0.05$ cm, as in the present work, the limiting oxygen consumption of the slice calculated using the above formula is 3.24 μl/hr·mg at 37°. Since the values of oxygen consumption observed in the present work are about 50% of this value (Table II), there is no reason to believe that acetate failed to increase oxygen consumption at 37° because of the diffusion limitation.

Whatever the mechanism of action of acetate is, one may rule out the possibility that acetate alters the affinity of the transport carrier (s) to *p*-aminohippurate, as indicated by the absence of any change in K_m in the presence of acetate (Fig. 2). The values of K_m measured in the present work at 37° agree well with those measured at 25° in the kidney cortical slice of the rabbit (6) and the rat (5). On the other hand, acetate significantly increased V_{max} in the present work. This finding is also compatible with an increase in $T_{M_{PAH}}$ *in vivo* in the dog kidney, following the administration of acetate (2). These results suggest that acetate increases the turnover rate of the carrier–organic acid complex, possibly by increasing its mobility.

The level of tissue adenosine triphosphate decreased significantly at 37° in the presence of acetate (Table III), although acetate

ABLE III. Effects of Acetate on Tissue Nucleotide Concentrations and Energy Charge Potential

	Milliosmolar concentration	Concentration of acetate in medium 10 m*M*	*P* value*
ne triphosphate (μmol/g wet wt)	1.74 ± 0.13[b]	1.40 ± 0.20	<0.05
ne diphosphate (μmol/g wet wt)	1.09 ± 0.10	1.44 ± 0.20	<0.05
ne monophosphate (μmol/g wet wt)	1.76 ± 0.28	1.88 ± 0.17	N.S.
:harge potential[c]	0.49 ± 0.03	0.44 ± 0.03	N.S.

dent's *t*-test (paired *t*-test) used for statistical comparisons.
ues are means ± SE of five observations.
TP] + ½ [ADP])/([ATP] + [ADP] + [AMP]).

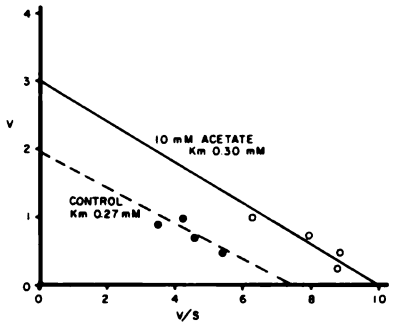

FIG. 2. Hofstee plot of kinetic data. The intercept on the ordinate represents the V_{max} of accumulation for either the control or a 10 m*M* acetate medium. The slope of the line represents the negative K_m. V_{max} and K_m are in millimoles/45 min/Kilogram wet weight and millimoles/liter, respectively.

did not increase the oxygen consumption at this temperature (*vide supra*). It is possible that acetate interacts specifically with adenosine triphosphate to energize the carrier–organic acid complex to increase its mobility. Since the metabolic rate is so high at 37°, there presumably is enough ATP in the tissue to support the action of acetate without increasing the oxygen consumption.

Based on these considerations, we propose that acetate enhances the active transport of *p*-aminohippurate by stimulating the utilization of cellular ATP. However, this utilization of ATP by the action of acetate does not disrupt the overall energy status of the transporting cells, as indicated by the absence of a change in the energy charge potential in the presence of acetate.

Summary. The present investigation involving *p*-aminohippurate uptake by rabbit kidney slices has shown (i) confirmative evidence that *p*-aminohippurate uptake is stimulated by the presence of acetate at both 25 and 37°; (ii) oxygen consumption being stimulated by acetate at 25 but not at 37°; (iii) the K_m for *p*-aminohippurate uptake at 37° is not affected by the presence of acetate in the medium; (iv) the V_{max} for *p*-aminohippurate uptake at 37° is directly related to the presence of acetate in the medium; and (v) acetate stimulates ATP utilization at 37° but does not alter energy charge potential.

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Received October 14, 1976. P.S.E.B.M. 1977, Vol. 154.

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Serum Gastrin in the Rat: Cholinergic and Adrenergic Effects[1] (39680)

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il recently neurochemical stimulation rin secretion has been considered to diated largely by cholinergic mecha- (1). However, in 1974 Kronborg *et al.* ported that a β-adrenergic blocking propranolol, could depress gastrin More direct evidence for adrenergic ement in gastrin secretion has ed from the following observations: tients with pheochromocytoma ex- l elevated blood gastrin levels which educed by either surgery or phenoxy- nine administration (3); (b) direct iv n of epinephrine in dogs (3) or man elevated blood gastrin levels; and (b) administration of a β-adrenergic ng agent, pindolol, eliminated the se- astrin stimulatory effects of iv epi- ne in normal subjects (5).

iously, we found pronounced effects drenergic drugs on both blood calcium yrocalcitonin secretion (6). Since se- n of gastrin and thyrocalcitonin appear lated in certain species (7), we de- to explore possible effects of β-adre- drugs on gastrin secretion. The find- esented here suggest that, in the rat, nergic amines can exert striking ef- n blood levels of immunoreactive gas-

erials and methods. *Animals.* Male nan rats (Madison, WI) 8-12 wk old eighing 200-400 g were used for all ments except that shown in Fig. 3 (32- old rats). Upon receipt they were ined on Purina Laboratory Chow and tap water. In each experiment, all rats were from a single shipment and had been born on the same day. For a period ranging from 18 to 48 hr before each experiment, food was withheld and animals were allowed only drinking water. In experiments involving feeding, Purina Chow pellets were offered after the fasting period, and the weight of food consumed was monitored.

Experimental procedures. Surgical procedures were conducted under ether anesthesia. Truncal vagotomy plus pyloroplasty were performed through a midventral incision. Both trunks of the vagus nerve were transected at the distal end of the esophagus, a 3-mm longitudinal incision was made at the pylorus through the mucosa, and the incision closed with 6-0 nylon sutures. Control rats were subjected to pyloroplasty only.

Injections were given either ip or iv (lateral tail vein) using a 27-gauge, 3/4-in. needle, and all injections were given in a total volume of either 0.5 or 1 ml. Control animals received injections of appropriate vols of vehicle (0.15 *M* NaCl or propylene glycol). Oral administration of drugs was by gavage using a 16-gauge curved metal feeding tube. The total vol of solution given by gavage ranged from 3-6 ml, and control rats received an appropriate vol of vehicle.

Test preparations. The drugs tested and their sources were as follows: *l*-epinephrine bitartrate and *l*-phenylephrine hydrochloride (Winthrop Laboratories, New York, N.Y.); d, *l*-isoproterenol hydrochloride (donated by Winthrop Labs); propranolol hydrochloride (donated by Ayerst Laboratories, New York, N.Y.); phenoxybenzamine hydrochloride (donated by Smith, Kline, and French Labs, Philadelphia, Pa.); atropine sulfate (Mallinckrodt Co., St. Louis, Mo.); acetylcholine bromide (Eastman Kodak Co., Rochester, N.Y.).

Most of the drugs were dissolved in 0.15

pported by U.S.P.H.S. Grants Nos. AM-10558 -17743 from the National Institute of Arthritis, lism and Digestive Diseases.

s study represents a part of work conducted by H. Hsu in partial fulfillment of the Ph.D. de- Pharmacology, U.N.C. School of Medicine.

.P.H.S. Career Development Awardee (AM-

1977 by the Society for Experimental Biology and Medicine erved.

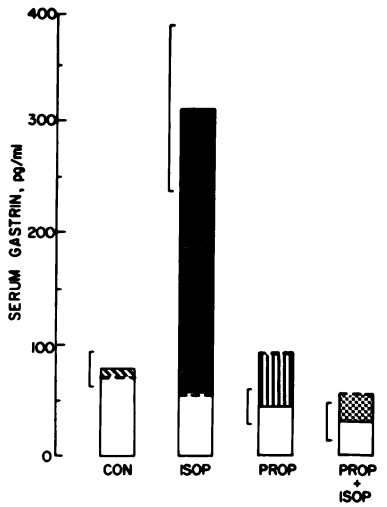
ISSN 0037-9727

M NaCl with the doses calculated on the basis of weight of salt; the dose of epinephrine was calculated on the basis of free base. Acetylcholine bromide was dissolved in distilled water; phyenoxybenzamine hydrochloride was dissolved in propylene glycol.

Blood collection and analyses. Either one or two blood samples (2 ml volume) were obtained from each rat either from the tail or by cardiac puncture. When two samples were obtained, one was taken at zero time before treatment and a second at the desired time interval after treatment. For analysis, sera were quickly obtained by centrifugation and kept frozen at −20° until assayed. Gastrin was determined using radioimmunoassay procedures described previously in detail (7, 8). A guinea pig antiserum to porcine gastrin (final dilution 1:100,000) was used, and synthetic 1-17 human gastrin (ICI, England) was used for both iodination with ^{125}I and as unlabeled reference standard. As reported previously (7), intra- and interassay variations in the range of serum gastrin measured in this study were no more than 15 and 20%, respectively.

Statistical analysis. In experiments where rats were fasted 24 hr or longer and some serum gastrin levels were below the limits of detectability of the immunoassay, individual values for each rat are presented; in these experiments, significance of differences between groups was assessed using the nonparametric test of Wilcoxon (9). In experiments where rats had been fasted for only 18 hr and all levels were detectable (Table I, Expt 2, and Fig. 1), the changes in gastrin for each group were subjected to analysis of variance, the standard errors were calculated from the residual error term of the appropriate analysis, and the significance of differences between groups was determined by the multiple comparisons test of Hartley (9).

Results. Table I, Expt 1, shows one representative experiment of several conducted where a very small dose of epinephrine (1 μg/kg iv over a 5-min period) was administered to intact rats. The results show that this low dose of epinephrine raised serum gastrin levels rapidly (within 10 min). Similarly, as shown in Expt 2, a significant ($P < 0.05$) increase in serum gastrin levels also



G. 1. Serum gastrin in rats injected with isopro-
ol (ISOP) or propranolol (PROP) alone or in
ination. Each bar represents serum gastrin from a
ate group of four to five rats. Each rat was bled
. Dashed lines with each bar show the mean 0 time
while the shaded areas show the 70-min change
rackets show the SE of the mean change. PROP
ng/kg) was given immediately after the initial
ling, and ISOP (1 mg/kg) was given 10 min later.
second bleeding was 60 min after ISOP. Control
eceived 0.15 *M* NaCl iv. Rats were 9 weeks old,
ed 250–300 g and had been fasted for only 18 hr.
ficant difference in changes: ISOP vs. Con or
P, $P < 0.05$; ISOP vs PROP + ISOP, $P < 0.02$;
P vs Con, $P < 0.05$.

of isoproterenol. In addition to block-
isoproterenol-induced increases in se-
gastrin (Fig. 1), injection of propranо-
lso restricted or prevented the rise in
m gastrin produced by feeding, as illus-
d in Fig. 2.

gure 3 illustrates that bathing the antral
osa with isoproterenol, like iv isoproter-
, also increased serum gastrin in the rat.
e, isoproterenol given by gavage to rats
the pylorus ligated resulted in a consist-
increase in gastrin 30 min later. Control
with the pylorus ligated and gavaged
0.15 *M* NaCl showed no demonstrable
ge in serum gastrin. In order to find out
ther the effect of isoproterenol was due
ncreased antral pH, isoproterenol was
lved in 0.01 *N* HCl and given by ga-
to rats with the pylorus ligated. Con-
rats received 0.01 *N* HCl only. As
vn in Table I, Expt 3 isoproterenol still

produced a large increase in serum gastrin after oral administration despite the fact that the pH of the stomach contents was kept low (pH = 2.0 ± 0.27) and did not differ significantly from that of control rats.

Possible interactions between cholinergic and adrenergic influences on serum gastrin responses in the rat were studied in the experiments shown in Figs. 4–6. As shown in Fig. 4, a large dose of acetylcholine given by gavage to rats produced a large increase in serum gastrin. In this experiment atropine, the postganglionic cholinergic blocking agent, given orally itself raised serum gas-

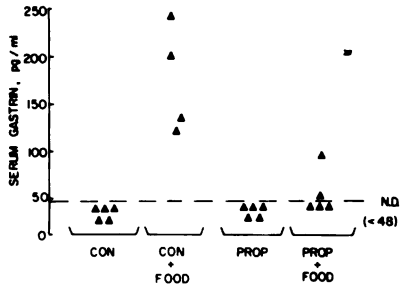


FIG. 2. Serum gastrin in rats pretreated with PROP and subjected to feeding. Each rat was fasted for 48 hr and then injected ip with 0.15 *M* NaCl (controls) or with PROP (10 mg/kg). Food (Purina Lab Chow) was presented immediately after PROP for a 1-hr period before blood collection; quantity of food consumed for all rats was 5.12 ± 0.35 g (mean ± SE). Each point represents an individual rat before or after feeding. Rats were 32 wk old and weighed 400–500 g. Significant difference: CON + Food vs CON, $P < 0.05$.

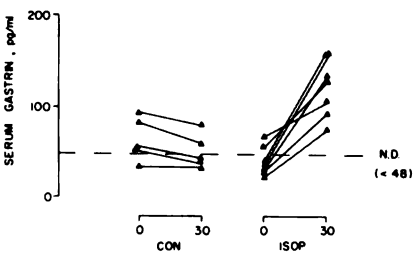


FIG. 3. Serum gastrin in rats with the pylorus ligated and gavaged with ISOP. Each rat was bled twice. After initial blood collection each rat underwent surgery and pyloric ligation. Then each animal was gavaged with 3 ml of 0.15 *M* NaCl (controls) or with ISOP (1 mg/kg in a 3-ml volume) and bled again 30 min later. Each pair of points represents an individual rat. Rats were 9 wk old, weighed 250–300 g and had been fasted for 24 hr. Significant change: ISOP vs CON, $P < 0.01$.

trin levels somewhat as reported by others (10) and, not unexpectedly, atropine also abolished or greatly restricted the ability of acetylcholine to increase serum gastrin. More surprisingly, a β-adrenergic blocking agent, propranolol, given orally, also appeared to reduce the serum gastrin response to acetylcholine, although two of the five rats tested still showed large responses to acetylcholine. Thus atropine restricted the response to acetylcholine, and propranolol also appeared effective in this regard (Fig. 4). Conversely, however, pretreatment of rats orally with atropine did not noticeably influence the serum gastrin elevation produced by administration of isoproterenol by gavage (Fig. 5).

The experiment illustrated in Fig. 6 shows basal (0 time) serum gastrin levels and 60 min responses to isoproterenol or propranolol in rats subjected to truncal vagotomy and pyloroplasty. Basal serum gastrin levels were highly variable in vagotomized rats but characteristically were higher than in control rats for all but three of 15 rats shown in Fig. 6. Injection of isoproterenol markedly raised the serum gastrin levels in vagotomized rats even further while injection of propranolol either reduced or did not affect

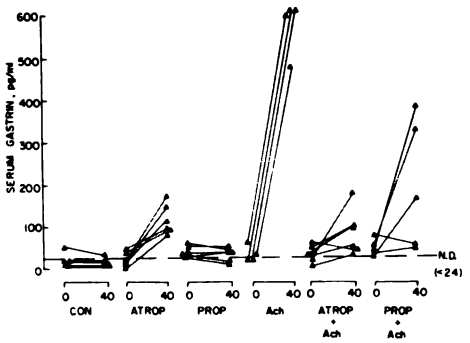


FIG. 4. Serum gastrin in rats gavaged with acetylcholine (Ach), atropine (ATROP) or PROP. Each rat was bled twice, once at 0 time and again 40 min later. ATROP, PROP (10 mg/kg in a 3-ml volume) or 3 ml 0.15 *M* NaCl (controls) was given immediately after initial blood collection; Ach (3 ml of 5% (w/v)) or vehicle (3 ml H_2O) was given 10 min later. Each pair of points represents an individual rat. Rats were 8 weeks old, weighed 220–280 g and had been fasted for 24 hr. Significant difference in changes: Ach or ATROP vs CON, $P < 0.01$; Ach vs ATROP + Ach or PROP + *Ach, P ≷ 0.01.*

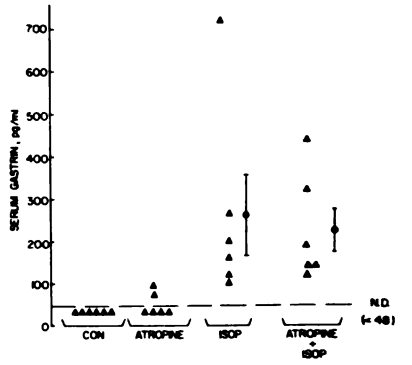


FIG. 5. Serum gastrin in rats gavaged with ISOP and ATROP alone or in combination. ATROPINE (10 mg/kg in 3 ml) or 3 ml 0.15 *M* NaCl (controls) was given and ISOP (1 mg/kg in 1.5 ml) or 1.5 ml 0.15 *M* NaCl was given 10 min later. Each point represents the serum gastrin level of an individual rat. Serum gastrin levels in both ISOP, and ATROPINE + ISOP are also presented as mean ± SE. Rats were 9 weeks old, weighed 220–320 g and had been fasted 48 hr. Significant difference: ISOP or ATROP + ISOP vs Con, $P < 0.05$; ISOP vs ATROP + ISOP, N.S.

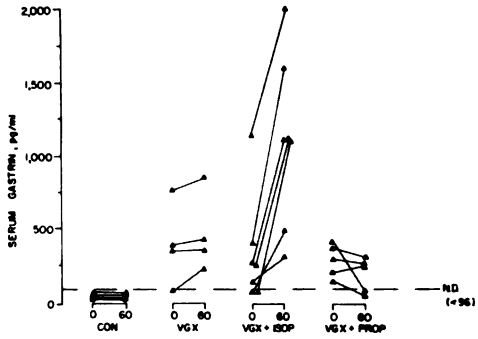


FIG. 6. Serum gastrin in rats after vagotomy (see Methods) and injection of ISOP or PROP. Vagotomy (VGX) was performed 30–60 days before the experiment. 0.15 *M* NaCl (controls), ISOP (1 mg/kg) or PROP (10 mg/kg) was injected immediately after bleeding at 0 time and rats were bled a second time 60 min later. Each pair of points represents an individual rat. Rats were 10–12 weeks old, weighed 250–325 g and had been fasted for 24 hr. Significant difference in changes: VGX + ISOP vs CON, VGX, or VGX + PROP, $P < 0.01$.

the elevated basal gastrin level in these animals.

Discussion. The present findings clearly show that in the rat catecholamines can markedly influence secretion of gastrin. In agreement with earlier observations by oth-

ıan and the dog (3–5), administra- ı small dose of epinephrine to rats d a detectable increase in serum (Table I). The catecholamine-in- ıcrease in serum gastrin appears to ated by β-adrenergic receptors. In of this idea are the present findings that (a) two β-agonists, isoprotere- epinephrine, raised serum gastrin able I, Expt 1 and 3; Figs 1 and 3) vagotomized rats with already high ıstrin levels (Fig. 6); and (b) a β- ʒic blocking agent, propranolol, d and hypergastrinemic effect of renol (Fig. 1) and reduced or abol- :reases in serum gastrin produced by (Fig. 2) and acetylcholine gavage . As reported for man (2), we also ł in one experiment (Fig. 1) that iv olol appeared to reduce even basal astrin levels. We found that an α- phenoxybenzamine, but not the α- phenylephrine, increased serum evels (Table I, Expt 2).

ffect of catecholamines on serum ıppears to be a direct one i.e., an rectly on the antral G-cell. In sup- his idea are the observations (a) that renol effectively increased blood ıfter introduction into the stomach with the pylorus ligated (Table I, Fig. 3); and (b) that parenteral ration of isoproterenol to acutely ɔmized rats did not increase serum unpublished results). The fact that : of the antral mucosa to isoprotere- n by gavage in 0.01 *N* HCl still serum gastrin also supports the ıt isoproterenol acted directly and suggested by earlier work showing ion of gastric acid secretion (11), y by elevation of intragastric pH. It rest that isoproterenol and epineph- e atropine (10), paradoxically can th inhibit gastric acid secretion (11) ncrease blood gastrin. This occurs he fact that the function of gastrin is ed to be promotion of gastric acid ı and cannot be explained by an in gastrin release secondary to a tragastric pH (Table I, Expt 3; 10). ınd Walsh (10) have suggested that dings with atropine alone may be best explained by the existence of cholinergic mechanisms which act to inhibit gastrin release as well as to promote gastric acid secretion. Similarly, β-adrenergic mechanisms may exist which act to promote gastrin release as well as to inhibit acid secretion. Whether endogenous catecholamines normally exert important effects on gastrin release in the rat remains to be established. Nevertheless, the present findings in the rat agree with earlier findings in the dog (3) and man (3–5) in suggesting that adrenergic mechanism may play a larger and more important role in mediating gastrin secretion than heretofore suspected.

Summary. Effects of vagotomy, acetylcholine and various adrenergic drugs on serum immunoreactive gastrin levels were studied in the rat. In rats fasted 24 hr or longer serum gastrin concentrations generally were <50 pg/ml; 60 min after the onset of feeding, serum gastrin concentrations reached levels >100 pg/ml. In rats fasted overnight, injections of epinephrine, isoproterenol and phenoxybenzamine all raised the serum gastrin level. Pretreatment with propranolol, a β-adrenergic blocking agent, abolished the hypergastrinemic affect of isoproterenol. Administration of propranolol also lowered basal serum gastrin levels and reduced the increase in serum gastrin produced by feeding. In vagotomized rats, isoproterenol futher increased the already elevated serum gastrin levels while propranolol alone slightly reduced the elevated basal gastrin levels in four or five vagotomized rats. Bathing the antral mucosa by gavage with isoproterenol (even in 0.01 *N* HCl) also increased serum gastrin levels, indicating that isoproterenol acted directly on the antrum and not indirectly by elevating intragastric pH.

Possible interactions between cholinergic and adrenergic agents on serum gastrin responses also were examined. Acetylcholine given by gavage produced its well known large increase in serum gastrin. An anticholinergic drug, atropine, given orally, greatly restricted the ability of acetylcholine to raise serum gastrin, and propranolol given orally also inhibited the increase in serum gastrin levels produced by oral acetylcholine. The findings support the idea that both choliner-

gic and adrenergic systems influence gastrin secretion in the rat.

The authors wish to thank Drs. Paul L. Munson, Philip F. Hirsch, Tai-Chan Peng, and Fred W. Ellis for advice during the course of this work. The technical assistance of Mr. Johnny F. Obie, Mrs. Deloris B. Alston, and Mr. John R. Hagaman, Jr. is deeply appreciated.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154

S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 407-411 (1977)

Evidence for a "Pro-relaxin" in Porcine Relaxin Concentrates (39681)

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ine relaxin has been characterized as l (5500–6000 dalton) basic protein of histidine, proline and tyrosine (1- ree different compounds with relaxin / have been isolated from relaxin con- es, using either ion-exchange chro- aphy (1) or column electrophoresis ese have similar biological activities 2500 GPU [guinea pig units (5)] per ıt differ in electrophoretic mobility ıino acid composition. Relaxin, like , consists of two peptide chains linked lfide bonds. An amino acid sequence : shorter (22 residue) chain has re- been proposed by Schwabe *et al.* (3). ıis paper, we present evidence for the ce of a larger molecule which may be ted to relaxin by exposure to trypsin. ıbstance, referred to here as 'prore- appears to possess intrinsic relaxin '.

rials and Methods. Relaxin, in the f an acid-acetone extract of ovaries of nt sows, was obtained from the Na- Institutes of Health. This material elaxin) contained 440 Warner-Lam- ıits/mg[1]. Bio Gel P-10 was obtained Bio-Rad Laboratories; it was equili- with NH_4OAc buffer, 0.10 *M*, pH ıd poured to form a column 1.84 × ı. Relaxin samples were dissolved in (25–100 mg/ml) and applied to the ı; 3–3.5 ml fractions were collected at e of 17–18 ml/hr. Cellex D (DEAE ıse), also obtained from Bio-Rad, ıuilibrated with 0.005 *M* NH_4OAc, ı and used in the form of a 1.0 × 57 umn. Samples were applied in the ɔuffer; after emergence of the first a linear gradient (0.005 → 0.50 *M* Ac, pH 7.0) was applied.

sample to be digested was dissolved *M* NH_4HCO_3 to a concentration of 25 mg/ml. Diphenyl carbamyl trypsin (DCC-trypsin, Sigma), also dissolved in 0.1 *M* NH_4HCO_3 was added in the proportion of 1 μg trypsin/mg substrate. A blank with the same amount of substrate, but containing no enzyme, was also prepared. After incubation at 37° for 30–60 min, the solutions were cooled to 0° and adjusted to pH 5.0 to terminate the action of the enzyme. They were stored frozen.

Disc gel electrophoresis was performed in 10% polyacrylamide gels using the technique described by Davis (6). The buffer [Tris chloride, pH 8.3] concentration in the electrode vessels was 0.05 *M* that in the gel was 0.025 *M*. After electrophoresis for 3 hr (5 ma/gel) the gels were fixed by immersion in 10% trichloroacetic acid for one-half hour, then stained overnight in 0.2% buffalo black. The gels were destained by immersion in MeOH:AcOH:H_2O (50:10:40).

Relaxin was assayed in guinea pigs primed with 5 μg estradiol benzoate (5). Each sample was tested at three or more twofold dilutions, using 9–10 animals for each concentration.

For molecular weight estimations, a P-10 column was calibrated using relaxin, cytochrome *c*, ribonuclease, ovalbumin, and bovine serum albumin as reference proteins.

Results. When NIH relaxin was chromatographed on Bio-Gel P-10, the protein emerged in two peaks, with V_e/V_t = 0.35 and 0.62, respectively (Fig. 1, upper panel). Of approximately 120,000 GPU of relaxin applied to the column, 6000 GPU (5%) appeared with the first peak (pool I, tubes 11–36), and 85,000 GPU (71%) was found in pool III (tubes 43–78). When material recovered from pool I was rechromatographed on the same column (Fig. 1, lower panel) all of the protein emerged in a single peak, with V_e/V_t = 0.35; this contained 80–90% of the activity (10,000 GPU) applied in this experiment, while only a small amount (~10%) was recovered from tubes

Warner–Lambert unit is approximately equal ıuinea pig unit" (5).

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ISSN 0037-9727

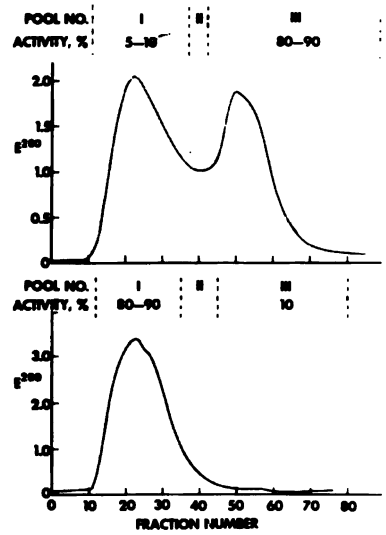
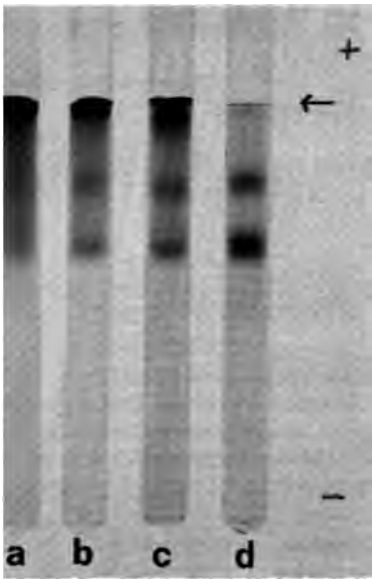


FIG. 1. Upper panel: Chromatogram of NIH relaxin (282 mg) on a 1.84 × 140 cm column of Bio-gel P-10. The buffer was 0.10 *M* NH_4OAc, pH 5.0. After collection of the first 62 ml of effluent (fraction 1) 3.5 ml fractions were collected. Lower panel: Rechromatography of 200 mg of pool I material, recovered from several experiments, on the same column. Fractions were pooled as indicated and assayed for relaxin activity.

45–80. These experiments suggested that the original preparation contained a substance with intrinsic relaxin activity which has a molecular weight significantly greater than relaxin itself.

Relaxin which has been purified by gel filtration displays a characteristic pattern when subjected to electrophoresis in polyacrylamide gels: two closely-spaced bands which migrate to the cathode at pH 9.0 or lower (Fig. 2, c). These have been identified (2) as proteins with nearly identical biological activities. At a comparable concentration (200 μg), the higher molecular weight material from pool I displayed no evidence for cationic components (Fig. 2, b); however, there appeared to be some diffusely staining material which migrated to the anode at pH 8.3. When this material was exposed to DCC-trypsin for 30–60 min two new components, corresponding in position and mobility to those found in gel-purified relaxin, appeared (Fig. 3, b and c). Furthermore, the relaxin activity of pool I, initially 65 ± 8 GPU/mg, was increased *after digestion* to 115 ± 20 GPU/mg[2].



Polyacrylamide gel electrophoresis of pool efore and after digestion with DCC-trypsin) 1 mg pool I, untreated; (b) 1 mg pool I trypsin, 37°; (c) 1 mg pool I after 60 min 200 μg NIH relaxin.

r method of separating relaxin presumptive precursor is by ion- chromatography on Cellex D (Fig. irst peak (I), which emerged with nt front, could be resolved into onic species, with electrophoretic corresponding to three active re- ies (Fig. 5, a). When a gradient of ionic strength was applied, two peaks appeared. Neither of these significant amounts of material toward the cathode at pH 8.3 and d). After treatment of mate- ered from peak II with DCC-tryp- min however, the electrophoretic vas characteristically relaxin-like :). On the other hand, no such ation occurred when peak III was o trypsin (Fig. 5, e).

out the possibility that noncova- ng of relaxin to a larger molecule for the experimental results de- ove, a sample of the material ob- m the first peak of a Bio-Gel P-10 ion of NIH relaxin was dissolved ea (in 0.1 *M* NH_4OAc buffer, pH llowed to remain at room temper- ature for 24 hr. This procedure resulted in no change in its electrophoretic behavior. Likewise, when pool I material was chromatographed on Bio-gel P-10 which had been equilibrated with 8 *M* urea in 0.1 *M* NH_4OAc, the profile of the chromatogram was indistinguishable from that observed in the absence of urea.

Discussion. The epxeriments summarized above indicate that porcine relaxin concentrates contain a constituent that can be converted to relaxin by digestion with DCC-trypsin. On the basis of its chromatographic behavior on Bio-gel P-10, the apparent molecular weight of the precursor is about 42,000.

Approximately 5–10% of the total activity of NIH relaxin is contained in the "prorelaxin" fraction recovered from Bio-gel; whether this represents an intrinsic property of the precursor or is due to its conversion to relaxin *in vivo* we do not know. Although some separation of "prorelaxin" from other, inactive components can apparently be achieved by ion-exchange chromatography on cellex D, we have so far been unable to resolve the high molecular weight fraction (Bio-Gel pool I) into discrete components by electrophoresis or other means. The relatively low yield of relaxin obtained after 30-min exposure to trypsin (equivalent to 3–4 mg from 50 mg pool I material) may be due to either insufficient exposure time, or the fact that NIH relaxin has been depleted in its pro-hormone content by the preliminary purification procedure.

Since the discovery of proinsulin by Steiner and Oyer in 1967 (7), high molecular weight biologically active or immunoreactive forms of several peptide or protein hormones have been identified in the plasma or endocrine tissues of several species of animals. These include adrenocorticotropin (ACTH) (8), gastrin (9), glucagon (10), growth hormone (11) and parathyroid hormone (PTH) (12). "Large" growth hormone appears to consist of growth hormone noncovalently bound to a larger molecule, since it can be converted to growth hormone by exposure to guanidine or by storage at −20°, while trypsin has no effect. The macromolecular forms of adrenocorticotropin ("big" ACTH), gastrin, and glucagon are

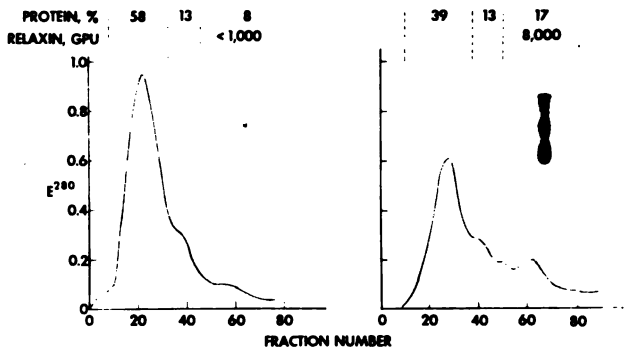


FIG. 4. Left panel: Chromatogram of 50 mg of untreated peak 1 material on Bio-gel P-10, 1.83 × 1 Right panel: Chromatogram of 50 mg of peak 1 material, after treatment with 50 μg. DCC-trypsin for 3(37°, on the same column. In both experiments 3.6 ml fractions were collected after the first 85 ml of efflu passed through the column. The effluent was pooled as indicated. The superimposed electropherograms a of 1.1 mg samples (estimated from E^{280} data) of corresponding pools from the two chromatograms.

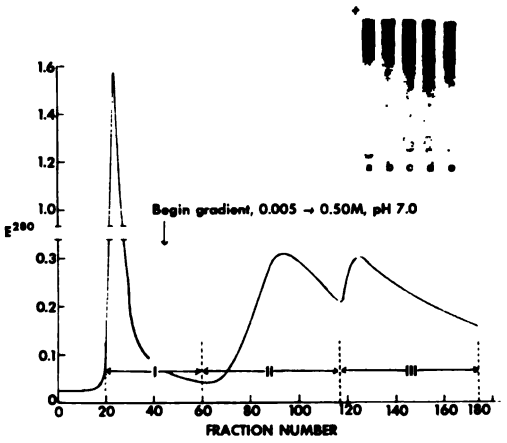


FIG. 5. Chromatogram of 50 mg NIH relaxin on Cellex D (1.0 × 57 cm). The sample was applied in 0.005 *M* NH_4OAc, pH 7.0, followed by a gradient to 0.5 *M*. In the upper right-hand corner are shown electropherograms of material recovered from the chromatogram. (a, b, d): pools I, II, III (untreated), respectively; (c, e) pools II and III, respectively, after 30' digestion with DCC-trypsin.

converted to the native hormones by digestion with trypsin, while the precursor form of parathyroid hormone is converted to PTH either *in situ* or *in vitro*, by trypsin. At present, the relation of relaxin to its presumed precursor seems to be similar to the case of 'big' ACTH, where the ACTH precursor has been reported to have a molecular weight between that of growth hormone and bovine serum albumin.

Summary. From 5 to 10% of the b cal activity of a porcine relaxin conce is contained in a fraction with a mo weight which is considerably (~42,000 daltons) than that of rela self. Exposure of this material to a lo centration of trypsin results in the a ance of native relaxin, as judged by crease in biological activity and char its chromatographic and electropl properties.

Presented at the 67th Annual Meeting of the can Society of Biological Chemists. San Fr California, June 6–10, 1976. From a thesis su by Li-An Yeh in partial fulfillment of the requi for the M.S. degree. Supported in part by a r grant from Hynson, Westcott, and Dunnin Baltimore, Maryland.

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Received July 19, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 412-414 (1977)

Fate of the Two Largest Follicles in the Ewe after Injection of Gonadotrophins at Two Stages of the Estrus Cycle (39682)

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During the estrus cycle ovulation can be induced in the ewe by either removing the corpus luteum (CL) (1, 2) or by injecting exogenous gonadotrophins (3-5). In these experiments it has not been determined however, whether the follicles that ovulated were the largest follicles at the time of treatment or that ovulation resulted from the rapid growth of smaller follicles.

In a previous experiment, Bherer *et al,* (6) reported that of the two largest follicles identified at Day 10 or Day 14 of the cycle in the ewe, only the largest follicle observed at Day 14 ovulated spontaneously at the next estrus. It was also observed that the removal of the CL when identifying the largest follicle at Day 10 of the cycle, did not at the ensuing estrus 2 days later, result in ovulation of the largest marked follicle. These findings were interpreted that the largest follicles present at mid-cycle were not responsive to the ovulating stimulus, or that this ovulating stimulus came too late or was not appropriate in bringing about ovulation of the largest follicle. It was also observed in these experiments that while the largest follicle marked on Day 14 lead to one ovulation, in cases of double ovulation, the second ovulation resulted from a smaller unmarked follicle.

The present experiment was designed to determine the ovulating capacity of the two largest follicles present at Day 10 or 14 of the estrus cycle with and without injection of exogenous gonadotrophins.

Materials and methods. Twenty crossbred ewes, 3 to 4 years of age, were checked daily for estrus (Day 0 of the cycle and of the experiment) with vasectomized rams and distributed at random to a 2 × 2 factorial experiment: the factors were (a) time of identification of the two largest follicles (Day 10 or day 14), and (b) type of injections (saline or saline and gonadotrophins).

For surgery, a general anesthetics (pentobarbital sodium) was used. The ovaries were exposed through a mid-ventral laparotomy. The largest and the second largest (F_1 and F_2, respectively) follicles were marked by injecting "dots" of India ink with a small syringe around the periphery of the follicle, just beneath the ovarian tunic and locations and diameters of the two largest follicles were recorded. One hour after recovery from anesthesia, the ewes were injected intravenously with 1 ml of Vetrophin or with 1 ml of saline. Vetrophin (Abbot Laboratories) is a sheep anterior pituitary extract; according to the manufacturer, one ml has an hormonal activity equivalent to 1 ml LH (NIH-LH-S1) + 1 mg FSH (NIH-FSH-S1).

On Day 19 of the experiment (i.e., 2 to 3 days after the postoperative estrus) the animals were autopsied. The ovaries were examined and a marked follicle was considered to have ovulated if the luteal structure was completely outlined by dots of India ink. Data were analyzed by the χ^2 method.

Results and discussion. The results obtained in the present experiment confirm previous observations (6) that marking of the follicles in the peripheral stroma does not interfere with ovulation of the marked follicles. Similar observation has also been done in cow (7) and Hunter and Baker (8) have shown that scratching and marking of the porcine follicle without piercing the follicle itself had no effect on ovulation.

The results presented in Table I show that in the five ewes laparotomized at Day 10 of the cycle, none of the marked largest follicles (F_1 and F_2) ovulated (Group I), although the follicles appeared similar to those found on Day 14 of the cycle and had the same diameter (7.0 ± 0.2 mm vs 7.3 + 0.3 mm). However, when gonadotrophins were injected immediately after marking at Day 10, a significant number of ewes (4/5) ovulated their largest follicle (Group II).

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ISSN 0037-97

ıg the three F_2 follicles which ovulated gonadotrophin injection, two were in ewes in which the F_1 follicles had vulated.

en marked on the 14th day of the the largest follicle (F_1) ovulated in ıut of five ewes which agrees with our ıus findings (6). When double ovula- ɔccured, the second ovulation never ɛd from the F_2 follicle. Injection of otrophins on Day 14 (Group IV) did ıange the proportion of F_1 ovulating but allowed 50% of the F_2 to ovulate. were found in ewes where the F_1 ɛ had also ovulated. Ovulation of the icle in Group IV appear to result from fect of exogenous gonadotrophin since fference in ovulation of F_2 follicle be- Group III and IV is significant at the evel and because spontaneous ovula- f the F_2 follicle identified at day 14 was encountered either in the present or ıus experiment.

ɔrding to Hay and Moor (9), follicles owing continuously and replacing each during the cycle. The data of the pres- periment thus suggest that the period ıe during which the F_2 grew from a diameter of 4.9 mm to become the t follicle (with a mean diameter of 7.3 is shorter than 4 days. It seems also he largest follicle seen at Day 14 can ɔn growing slowly for at least 2 days ɛ becoming a much larger preovula- ɔllicle on the day of estrus; Brand and ng (10) and Hutchinson and Robert-

LE I. FATE OF THE TWO LARGEST FOLLICLES /E AFTER VETROPHINE INJECTIONS ON DAY 10 OR 14 OF THE ESTROUS CYCLE.

Vetrophin (1 ml)[a]	Time of marking and of hormone injection	Ovulated follicle[b]		
		F_1	F_2	F_u
–	10	0/5	0/5	6
+	10	4/5[d]	3/5[d]	4
–	14	4/5[d]	0/5[e]	3
+	14	3/4	2/4	4

ıe milliliter was equivalent to 1 mg LH (NIH- + 1 mg FSH (NIH-FSH-S1).

largest follicle; F_2, second largest follicle; F_u, ed follicle.

mber of ewes per group.

nificantly different ($P < 0.05$) from corre- g value of Group I.

nificantly different ($P < 0.05$) from F_1 value.

son (11) have shown that there was very little change in the diameter of the largest follicle during the last days of the cycle.

While in another study (6) the largest follicles (F_1 and F_2) present at the 10th day of the cycle could not be ovulated by CL removal, the present experiment shows that these follicles can be ovulated by exogenous gonadotrophins. These two studies do suggest that the largest follicle (F_1) present at mid-cycle is not as responsive as the largest follicle present at the end of the cycle, but that it can be ovulated if properly stimulated and that exogenous gonadotrophins are a more appropriate stimulus than CL removal in making the largest follicle of day 10 ovulate.

The present results also agree with previous observations that F_2 follicles of the 14th day of the normal cycle do not ovulate spontaneously (Group III) and that the second ovulation in ewes having a spontaneous double ovulation came from smaller unmarked follicles.

This raises the question as to why F_2 follicles present on Day 14 do not ovulate spontaneously at the next estrus. The F_2 follicles at Day 14, as well as at Day 10, probably represent a mixed population: actively growing follicles on one hand and on the other hand former F_1 follicles which are undergoing atresia and are regressing. If some of the F_2 follicles are regressing, it is not surprising that they do not ovulate at the next estrus. However it is possible, as suggested by Hay and Moor (9), that in some of these follicles, atresia could be stopped and reversed by the gonadotrophin injection so that some of them would ovulate. However this does not explain why among the F_2 follicles present at day 14, those which are actively growing do not ovulate spontaneously and are replaced by smaller follicles. We have already advanced the hypothesis (6) that the F_1 follicle could exert an inhibiting influence on the F_2 follicle. Although the present experiment was not devised to prove this hypothesis, it seems to support the idea that the F_2 follicles of day 14 do not normally ovulate and that they require an extra stimulus to do so.

Summary. The response of the two largest follicles, identified at Day 10 or Day 14 of the estrous cycle in ewe, was determined by

injecting exogenous gonadotrophins. The largest follicle marked on the 10th day of the estrus cycle ovulated only when the ewes were given gonadotrophins (control 0/5; treated 4/5), while when marked on the 14th day, it did not require gonadotrophin injection to ovulate (control 4/5; treated 3/4). The second largest follicle marked on the 10th or 14th day ovulated in 60% and 50% of the treated ewes respectively, while nontreated ewes did not ovulate any of the second largest follicles at the ensuing estrus.

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Received September 1, 1976. P.S.E.B.M. 197 154.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 415–417 (1977)

ıe Effect of Stress on the Gastric Mucosal Barrier in Rats[1] (39683)

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ric mucosa presents a barrier to iffusion of hydrogen ions (H^+) gastric lumen (1, 2). Such sub-ɔile (3), weak acids (4), and acet- (2) may alter and increase the ty of this barrier resulting in in-:k-diffusion of H^+ ions and subse-e damage. Critically ill man has H^+ back-diffusion across the gas-ı (5). Experimental shock in dogs astric erosions, but an increase in :y to H^+ has not been demon-). Rats subjected to restraint-lop ulceration of the stomach (7, :ir gastric secretory rates are re-ıarkedly decreased (9); however, ıcid back-diffusion has not been ed (10). In order to reevaluate H^+ back-diffusion in restrained ıdified previously described tech- small animals by adapting meth-ı this laboratory in dogs and in (3, 11). The findings presented cate that increased rates of H^+ ion occur across the gastric mu-:ssed rats.

Eighteen male Sprague-Dawley ng 250–300 g were used. Nine mals were fasted for 48 hr with to water in raised mesh-bottom ɔtain maximal gastric emptying. ı be restrained were fasted for 24 ere restrained in wire cages for two supplements of 2.5 ml of ım chloride were given subcuta- each rat. Both control and re-:s were anesthetized with intra-ɔentobarbital (4 mg/100 g body l maintained under anesthesia by mall quantities of pentobarbital ıeal as needed. Nonvagotomized ıted stomachs were prepared lae in the esophagus (12). The ıs washed gently with 2 ml of isotonic solution containing HCl 55 meq/liter and NaCl 100 meq/liter. The test solution was identical to the wash solution but contained phenol red (10 mg/liter) as indicator.

Acid instillation was performed similarly to our previous canine instillation experiments (3) with slight modifications. For greater accuracy all solutions were weighed. A 10-ml syringe containing 8 ml of test solution was attached to the cannula in the esophagus. Four ml of fluid were instilled into the stomach and then removed and mixed with the remaining 4 ml to determine residual volume. This was repeated twice. Four milliliters were used as the instillation test solution; the remaining 4 ml was weighed and used as an initial aliquot. At the end of 1 hr, the gastric contents were removed and weighed. If the animal's condition was satisfactory, another instillation was performed. No more than three instillations per rat were done because of the risk of ulcerations in such preparations after 4–6 hr (13). Preliminary experiments had revealed no ulcerations developed in 3 hr-pylorus-ligated rats. Hydrogen concentration was determined by titration to pH 7.0 using a Radiometer electrometer titrator. Sodium and potassium concentration were measured by flame photometer using lithium internal standards. Phenol red was determined by spectrophotometer (14).

Ionic fluxes, residual volumes, hydrogen secreted and hydrogen neutralized were determined using assumptions previously described by us and others utilizing the "two component hypothesis" (5, 10, 11). Statistical significance of means was determined by Student's *t* test.

Ulcerations were graded on a scale of 1 to 4 by gross visual inspection: 1, one ulceration; 2, two to three ulcerations; 3, three to eight ulcerations; 4, more than eight ulcerations.

Results. H^+ and sodium fluxes are pre-

l by NCI grant No. 1 R01 CA15541-01.

by the Society for Experimental Biology and Medicine

ISSN 0037

sented in Table I. Restrained rats had a highly significant ($P < 0.001$) increase in the rate of hydrogen ion back-diffusion across the gastric mucosa. Restrained animals did not secrete significantly less acid than control animals. The amount of H^+ neutralized was similar in both groups. Sodium fluxes were similar in control and restrained rats.

Twenty-four hours of restraint produced varying amounts of gastric erosions in all nine restrained animals; no lesions were observed in control animals.

There was a high correlation ($r = +0.88$) between the severity of erosions and the magnitude of H^+ ion back-diffusion in the restrained rats (Table II).

Discussion. Our results indicate that restraint stress in rats increases the rate of back-diffusion of H^+ across the gastric mucosa. This increase correlated well with the severity of the induced mucosal ulceration. This increased permeability of the gastric mucosa may explain some of the published results on hypoacidity of restrained rats (9).

The acid-instillation technique for measuring ionic fluxes across the gastric mucosa in small animals utilized in this study has several advantages over previous techniques (12) in that it allows one to measure residual volume and both parietal cell and nonparietal secretion. In one previously utilized method (12), initial ion values are assumed to be those of the stock solution. Our initial ion values are determined by an aliquot obtained after washing the gastric mucosa with test solution. The drop in H^+ and phenol red concentration from the stock solution to the initial aliquot at times was as high as 20%. Furthermore, instillate volumes of 8 ml in 300-g rats were used by others (12); we found that such a volume produced tension in the gastric wall with intragastric pressure of 24 cm/H_2O. Our volume of 4 ml more

TABLE I. IONIC FLUXES DURING ACID INSTILLAT

	H+ Back-diffusion (μeq/hr)	Hydrog (μ
Control $n = 22$	9.2 ± 11.0	33.8
Restrained $n = 25$	−47.3 ± 7.2[a,c]	20.0

[a] $P < 0.001$.
[b] $P < 0.2$
[c] *Minus sign signifies* flux of ion from lumen to muco

TABLE II. MAGNITUDE OF H+ IN RESTRAINED RATS WITH DIFFERENT DEGREES OF ULCERATION.

Severity of ulceration	Number	H+ Back-diffusion
Erythema	5	$-10.65 \pm 9.73^{a,b}$
1	3	-42.79 ± 7.96^{c}
2	6	-40.36 ± 7.87^{d}
3	6	-70.74 ± 17.19
4	5	-66.10 ± 17.13

[a] Erythema vs 1 or 2. $P < 0.005$.
[b] Erythema vs 3 or 4. $P < 0.001$.
[c] One vs 3 or 4. $P < 0.01$.
[d] Two vs 3 or 4. $P < 0.01$.

Hase (18, 19) showed that stressed rats with ulcerations had greater acid secretion than stressed rats without ulceration. Back-diffusion was not determined in these studies. We show no difference in rates of acid secretion. The importance of intraluminal acid in the pathogenesis of restraint ulcers is further demonstrated by the observation that vagotomy and anticholinergics decrease the severity of stress induced ulcers (9, 20). Our studies indicate that back-diffusion of secreted acid may be involved in the restraint induced mucosal ulcerations.

Summary. To obtain evidence about the controversial role of the gastric mucosal barrier to back-diffusion of hydrogen ions in the pathogenesis of gastric ulcerations during restraint-stress, rats were subjected to restraint stress for 24 hr and acid instillations performed in their pylorus ligated stomachs with a technique adapted from previously described methods for acid instillation in canine dog pouches. Since the rat's pylorus ligated stomach does not tolerate solutions with high hydrogen ion (H^+) concentrations, test solutions containing H^+ of 55 meq/liter were used. Hydrogen ion secretion did not decrease in these stressed rats, but there was a marked increase in H^+ back-diffusion across the gastric mucosa which was well correlated with the gross visual damage. Abnormal gastric mucosal permeability to hydrogen ion may play a pathogenic role in restraint-stress induced ulceration.

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Received October 17, 1976. P.S.E.B.M. 1977, Vol. 154.

Chlorination by the Myeloperoxidase-H_2O Neutral pH

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The enzyme, myeloperoxidase (MPO), has been widely studied. However, it was not until recently that information relative to its biological significance has become available. MPO, in the presence of H_2O_2 and a halide has been found to constitute a powerful antimicrobial system (1, 2). Since polymorphonuclear leukocytes (PMN) appear to be the only source of this enzyme, and since a primary function of these phagocytes is to destroy engulfed microbes, it is reasonable to postulate that the physiological function of MPO is its participation in host-defense mechanisms. With chloride as the halide, MPO has been found to catalyze the oxidation of Cl^- ion by H_2O_2 to yield the chlorinating agent, the chlorinium ion (Cl^+) (3, 4). The antimicrobial activity of the MPO-H_2O_2-Cl^- system is probably due to the formation of this chlorinium ion. The Cl^+ ion can react with substrates such as bacterial cell walls, (5), amines, (4), amino acids (3) and other substrates (5, 6) resulting in chlorination and antimicrobial activity.

It has been reported that the optimal pH for chlorination, decarboxylation and bactericidal activity is 5.5 (1). Although it has been suggested that the pH can drop to this level in the phagocytic vacuole, (7, 8), there is no definitive proof that this pH is actually reached in the human PMN. Interestingly, it has been found that chlorination and decarboxylation can occur optimally at a pH range of 3.6–5.0 providing the chloride concentration is changed from 10 to 400 m*M*, respectively. This observation indicates that a relationship between [Cl^-] and optimal pH exists (4). Similar to H_2O_2, Cl^- is also known to be bound to Fe-porphyrin groups

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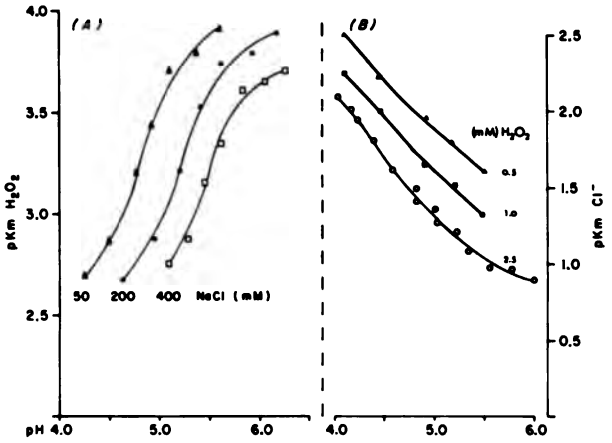


3. 1. (A) Influence of pH and Cl^- on apparent affinity between myeloperoxidase and H_2O_2. (B) Influence I and H_2O_2 on apparent affinity between myeloperoxidase and Cl^-. The velocity of diethanolchloramine ıtion at 20° was measured as increase in absorbance at 265 nm. Reaction mixture contained 10 m*M* citrate ›hate buffer at the indicated pH and different concentrations of H_2O_2 and Cl^-. K_m values for chloride and were calculated using Lineweaver-Burk plots ($pkm = -\log K_m$). (A) Cl^- concentrations 50 (▲——▲), 200 —●), 400 (□——□) m*M*. Five different H_2O_2 concentrations from 0.1 to 5.0 m*M* were used for any imental point. (B) H_2O_2 concentrations 0.5 (▲——▲), 1.0 (■——■), and 2.5 (○——○) m*M*. Five different concentrations from 2.5 to 200 m*M* were used for any experimental point.

ɪrent Cl^- concentrations can also be ex- ed to affect MPO-H_2O_2 affinity. Exper- ıts similar to those above were designed ɛtermine the correctness of this hypoth-

The H_2O_2 concentration was varied ı 0.1 to 5 m*M*. The Cl^- concentrations l were 50, 100, 200, 300, and 400 m*M*. sake of clarity, only 50, 200, and 400 data were shown. As expected, Cl^- ion ɛntration affected the apparent affinity ɾeen MPO and H_2O_2. Increasing con- rations of Cl^- ion decreased MPO- $_2$ affinity. Similar to MPO-Cl^- affinity, O-H_2O_2 affinity is also pH dependent.

ıe observed influence of pH on affinity ɪionships between MPO and H_2O_2 and O and Cl^- led us to expect different mum pH values for MPO mediated rination with different concentrations of $_2$ and Cl^-. An experiment to test this ›thesis was performed and the results be seen in Fig. 2. It is evident that there ɔ fixed optimum pH for chlorination. ıer, optimal chlorination can occur over ide pH range. For instance, one may in optimal chlorination between pH 4.0 6.0 by simply adjusting the Cl^- and $_2$ concentrations.

ɔm the above results, one can see that *H_2O_2]/[Cl^-] ratio is critical* for the opti-

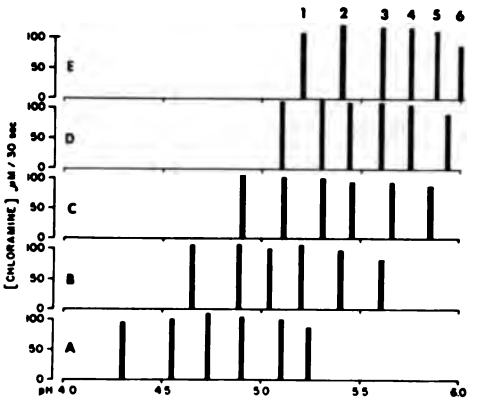


FIG. 2. Influence of H_2O_2 and Cl^- concentrations on optimal pH for myeloperoxidase mediated chlorination. Experimental details were as described for Fig. 1. Cl^- concentrations (m*M*): 50 (A), 100 (B), 200 (C), 300 (D), 400 (E). H_2O_2 concentrations (m*M*): 1.667 (1), 0.833 (2), 0.667 (3), 0.5 (4), 0.333 (5) and 0.167 (6).

mal velocity of chlorination for any given pH. The relationship between pH and the required $[H_2O_2]/[Cl^-]$ ratio for optimal chlorination may be seen in Fig. 3. It is evident that pH changes are proportional to changes of log $[H_2O_2]/[Cl^-]$. Since pH is a logarithmic function of H^+ concentration, the experimentally obtained curve may be expressed by a single equation: $K = [H_2O_2]/$

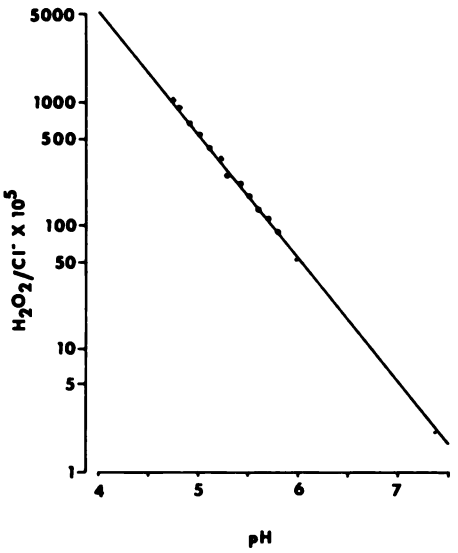


FIG. 3. Optimal pH for myeloperoxidase mediated chlorination at different H_2O_2/NaCl ratios. Data calculated from results presented in Fig. 2.

$[Cl^-]/[H^+]$, where K is the proportionally coefficient and is experimentally found to be equal to 560. A change in this value will result in a decrease of velocity. For example, if the $[H_2O_2]/[Cl^-][H^+]$ ratio is 250 or 1250, the velocity of the reaction was found to be 75% of optimal.

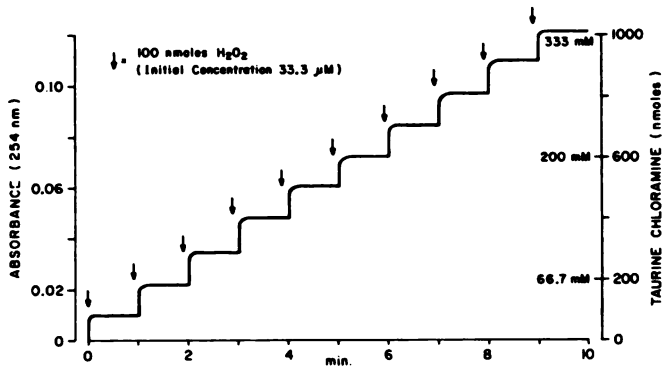
The relationship between Cl^- and H_2O_2 concentrations and pH was experimentally established for pH values between 4 and 6. Since the relationship of pH and log $[H_2O_2]/[Cl^-]$ ratio is a straight line, extrapolation of optimal condition for pH values higher than pH 6 is theoretically possible. It would be of interest to see if extrapolation of the curve to neutral pH can be experimentally confirmed.

From the above formula, for pH 7.4 and 150 mM Cl^- ion concentration, optimal concentration of H_2O_2 should be 3.4 μM. This amount of H_2O_2, even after complete conversion to chloramine, will have an absorbance of 0.0012 for a 1 cm light path. Such low values cannot be accurately measured. Hence, in chlorination experiments at pH 7.4, H_2O_2 was added to achieve 10 times higher than optimal concentration and in multiple steps; sodium chloride concentration was also increased to 400 mM. Under these experimental conditions, the velocity of the reaction should be 50–75% of the optimal velocity predicted by the formula. By doing this, sufficient accumulation of product for accurate measurement was obtained in spite of the fact that low amounts of H_2O_2 were used. The results of this experiment may be seen in Fig. 4. This indicates that chlorination occurs efficiently at pH 7.4. It may be noted that an immediate conversion of H_2O_2 to product occurs within seconds. Further, the conversion is stoichiometric. It is obvious from these data that extrapolation of the experimental data shown in Fig. 3 can be made correctly.

Discussion. There exist numerous reports that at acidic pH, MPO-H_2O_2-Cl^- system is bactericidal to a variety of bacteria, fungi, mycoplasma, and virus. It was of interest to ascertain, whether such a universal microbicidal system may also be able to function at a wide variety of physiological conditions such as varying pH, $[H_2O_2]$ and $[Cl^-]$ that may exist in the granulocyte. We have approached this problem by investigating the apparent affinity relationship between MPO-H_2O_2 and MPO-Cl^- at different Cl^- and H_2O_2 concentrations, respectively. Also, the influence of pH on these parameters was examined. Although multireactant enzyme kinetics are complex, Cleland (10) has shown that initial velocity analysis suffices for Michaelis constants and maximum velocities. Most of our kinetic data were obtained by measuring the initial velocity of the reaction.

Results presented in Fig. 1 show that the apparent affinity of MPO to Cl^- varies with pH and/or $[H_2O_2]$. Higher $[H^+]$ or lower $[H_2O_2]$ leads to an increase in the affinity of MPO to Cl^-. Somewhat similar to our findings, Hager *et al.* (11) have reported that the affinity of chloroperoxidase to Cl^- also varies with pH and changes in the same direction as in MPO. However, H_2O_2 does not affect chloroperoxidase-Cl^- affinity. Similarly, the apparent affinity of MPO to H_2O_2 also varies with pH and/or $[Cl^-]$. This property of MPO appears to be unique among all enzymes utilizing H_2O_2 substrate. Even chloroperoxidase, which is very similar to MPO in its mechanism of chlorination does not show variable affinity toward H_2O_2 (11).

In addition, by examining V_{max} values from Lineweaver-Burk plots (data not



Myeloperoxidase mediated taurine chlorination at pH 7.4. Reaction mixture contains: 20 m*M*) m*M* NaCl, 10 μg of MPO/ml, 100 m*M* citrate phosphate buffer at pH 7.4. Reaction was started by ı of 5 μl of 20 m*M* H_2O_2 (arrows) to obtain temporal concentration of H_2O_2 in the sample (3 ml) equal Chlorination was measured as absorbance increase at 254 nm at 20°.

ne can note the competitive nature H_2O_2 and Cl^- ion, H_2O_2 and H^+ and OH^-. This is, of course, true he V_{max} in each instance is similar These results further indicate that and H_2O_2 bind to the same locus of ırther, these bindings most proba- in the Fe-porphyrin nucleus, since e spectral changes in the Soret re- ce varying, at constant ratio, the ıtions of two substrates (H_2O_2/Cl^-) optimal velocity of chlorination for pH, a Ping Pong mechanism (12) operating. From a physiological view, this may be of significance. re, the MPO-H_2O_2-Cl^- antimicro- n was reported to be effective only H. Since controversy exists as to he pH drops intracellularly to 5.0 cells, a microbicidal system func- neutral pH values would be desir-

be concluded from this study, that)-H_2O_2-Cl^- antimicrobial system ınctional over a wide range of pH .0-7.4), H_2O_2 and Cl^- ion concen- It is no longer necessary to associ- :tivity of this system strictly to the I. A subsequent publication will t the MPO-H_2O_2-Cl^- system is lal at pH 7.4.

ıese and previous kinetic studies of is apparent that this enzyme is t has at least two different primary ; (i.e., H_2O_2 and Cl^-), it has a optimum (4.0-7.4) depending on ive concentrations of these sub- strates, and, finally these substrates compete for the same site of the enzyme. All these properties are compatible for MPO activity over a broad biological range.

Summary. The oxidation of Cl^- ion by H_2O_2 yielding Cl^+ is catalyzed by myeloperoxidase (MPO) prepared from human PMN. Both H_2O_2 and Cl^- in this reaction serve as substrates. Affinity (apparent) studies between MPO and Cl^- and MPO and H_2O_2 were carried out. Both (apparent) affinities vary according to pH. The higher the pH, the less the affinity for Cl^-. In contrast, the affinity between MPO and H_2O_2 increases with pH. The dissociation constant of the MPO-Cl^- enzyme substrate complex is affected by the second substrate, H_2O_2. The effect of H_2O_2 on the MPO-Cl^- affinity is competitive. Likewise, the MPO-H_2O_2 affinity is decreased with increasing Cl^- concentration. This influence is also competitive. From these interactions, it may be concluded that the optimal pH for chlorination is dependent on the $[H_2O_2]/[Cl^-]$ ratio. The relationship of pH, H_2O_2, and Cl^- concentrations may be expressed by the following formula: pH = log $[Cl^-]$ ×560/ $[H_2O_2]$. Using the formula, it can be shown that optimal chlorination may occur between pH 4.0 and 7.4.

We wish to thank Paul Wallace and Gerry Heatley for technical assistance, Roberta Lemon for preparing the manuscript, and George Daynes for photography. This investigation was supported by Grant CA-1567 awarded by the National Cancer Institute, DHEW, and USPHS Grant HD-1805, NIH.

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Received May 20, 1976. P.S.E.B.M. 1977, Vol. 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 423-426 (1977)

ɔdynamic Factors and Vascular Density as Potential Determinants of Blood Flow in Hypertrophied Rat Heart (39685)

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n a chronic pressure overload is im-
ıpon the heart, additional myocardial
ıccumulates as a compensatory re-
. The additional heart mass creates
ed tissue oxygen demands which, un-
rmal circumstances, would be met
ly by increasing coronary blood flow
owever, recent studies have shown
:al coronary blood flow (2) and flow
t mass of tissue (3) are reduced be-
rmal levels in hypertrophied hearts.
apparent failure to meet the addi-
ıxygen demands of the hypertrophied
ıay be related to hemodynamic deter-
ɜ of coronary blood flow (4) which
ıany the pressure overload. Alterna-
coronary vascularity may fail to in-
in proportion to the increase in my-
l mass. Since blood flow to the endo-
portion of the left ventricle is consid-
be marginally adequate under nor-
ıditions (5, 6), it seemed likely that
tion of the ventricle would be partic-
susceptible to pressure overload

efore, hemodynamic factors related
ɔnary blood flow regulation were
ed in control and aortic constricted
lkaline phosphatase specific activity
ized as a quantitative chemical index
ular density in the epicardial (EPI)
docardial (ENDO) portions of con-
l hypertrophied left ventricles. In this
ıe relative potential contribution of
ɾnamic factors and/or coronary vas-
to blood flow regulation in the hy-
hied heart could be evaluated.

ɾials and Methods. Animals and surgcedures. Male Sprague-Dawley rats
ıg 250–275 g were used in these ex-
ıts. Pressure-induced left ventricular
ypertrophy was created by constrict-
abdominal aorta above the renal
(7). Control animals received sham
ɔns. Aortic constricted and control animals were studied 3 weeks following surgery at which time stable LV hypertrophy would be expected in aortic constricted animals (8).

Hemodynamic measurements. Animals were anesthetized with sodium pentobarbital (approx 50 mg/kg, ip), weighed and placed on positive pressure ventilation with room air via a tracheostomy. LV pressure, heart rate, mean arterial pressure, and the maximum rate of LV pressure development (max *dP/dt*) were measured as previously described (9). The max *dP/dt* was utilized as an index of myocardial contractility. After hemodynamic measurements were completed, the heart was excised and placed into a beaker of ice-cold 10 m*M* Tris-saline buffer, pH 7.4.

Tissue preparation. The excised heart was cleared of blood by perfusing 7 ml Tris-saline buffer retrograde through the aorta. Atria, great vessels and the right ventricle were then carefully removed. The remaining LV plus interventricular septum was separated into EPI and ENDO portions by dissecting the ventricle at the midwall. These tissue samples were weighed separately and the combined values were taken as LV weight.

Chemical analyses. In the rat heart, alkaline phosphatase has been histochemically localized primarily in coronary vessels and capillary endothelial cells (10, 11). Therefore, the chemical determination of alkaline phosphatase specific activity in heart tissue was utilized as a quantitative estimation of vascular density. EPI and ENDO portions of the LV from one group of animals were homogenized in Tris-saline buffer using a ground-glass homogenizer. Following centrifugation at 27,000*g* for 20 min, the supernatant was removed and alkaline phosphatase specific activity was determined (12). Supernatant protein was measured by the biuret reaction. Enzyme activity was deter-

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ISSN 0037-97

mined under linear conditions with respect to incubation time and enzyme concentration.

In another group of animals, a 10% (w/v) homogenate of each LV portion (EPI and ENDO) was prepared using ice-cold distilled water. Aliquots were analyzed for protein, RNA, DNA, and hydroxyproline as previously described (13).

Statistical methods. Within groups, EPI and ENDO values were compared using Student's paired *t* analysis. Comparisons between control and aortic constricted animals were made by unpaired *t* analysis. A *P* value of 0.05 or less was considered statistically significant.

Results. At the time of sacrifice, the combined control animals used in this study weighed 384 ± 9 g (mean ± SE). Since the body weights of aortic constricted animals were similar to those of control animals (374 ± 8 g), the efficacy of aortic constriction in producing hypertrophy could be evaluated without resorting to body weight normalizing procedures. LV weight was 792 ± 22 mg in control animals and was significantly increased to 981 ± 40 mg in aortic constricted animals.

The hemodynamic measurements obtained from control and aortic constricted animals are shown in Table I. Abdominal aortic constriction produced a substantial LV pressure overload, as evidenced by a 40 mm Hg elevation in peak LV pressure. In addition, significant increases in heart rate, mean arterial pressure and max *dP/dt* accompanied the LV pressure overload.

The alkaline phosphatase results are shown in Table II. In control animals, the

TABLE I. HEMODYNAMIC MEASUREMENTS FROM CONTROL AND AORTIC CONSTRICTED ANIMALS[a]

	Control (9)	Aortic constricted (15)
LVP[b], mmHg	120 ± 4	162 ± 5[d]
Heart Rate, bpm	398 ± 8	438 ± 9[d]
MAP[c], mmHg	104 ± 4	118 ± 4[d]
Max. dP/dt, mm Hg/sec	6550 ± 350	8420 ± 340[d]

[a] Values are mean ± SE with number of animals in parentheses.
[b] Peak left ventricular pressure.
[c] Mean arterial pressure.
[d] $P < 0.05$ *vs* control.

LE III. PROTEIN, NUCLEIC ACIDS AND CONNECTIVE TISSUE LEVELS IN EPICARDIAL AND ENDOCARDIAL PORTIONS OF LEFT VENTRICLE FROM CONTROL AND AORTIC CONSTRICTED ANIMALS.[a]

	Control (11)			Aortic constricted (8)		
	Epi[b]	Endo[c]	Endo/Epi[d]	Epi	Endo	Endo/Epi
in	185 ± 7	176 ± 7	0.95 ± .02	176 ± 10	161 ± 5	0.92 ± .03
	3.72 ± .11	3.53 ± .14	0.95 ± .02	3.67 ± .09	3.53 ± .09	0.96 ± .02
	2.76 ± .10	2.43 ± .10	0.88 ± .03	2.90 ± .23	2.68 ± .29	0.92 ± .06
*	0.89 ± .03	0.81 ± .04	0.92 ± .04	0.92 ± .05	0.92 ± .04	1.00 ± .07

tissue level values are mg/g wet weight with mean ± SE shown. Number of animals given in parentheses.
icardium.
docardium.
tio of endocardial-epicardial tissue levels.
droxyproline.

ed from aortic constricted rats in the
it study, it is clear that elevations in
essure, heart rate, max. *dP/dt* and
ad also dictate increased myocardial
n consumption (4). The heart nor-
extracts all but approximately 5 vol%
ilable oxygen from arterial coronary
(1, 14). Since little additional oxygen
: obtained by increasing oxygen ex-
n, coronary blood flow would be ex-
to increase in order to supply the
onal oxygen required by the hypertro-
LV. However, recent studies have
that total blood flow to the hypertro-
LV (2) as well as blood flow/g tissue
reduced below normal levels.
ombination of multiple hemodynamic
; influences net coronary blood flow
ne elevated mean arterial pressure ob-
in aortic constricted rats would in-
coronary perfusion pressure and tend
oke a similar directional change in
flow. In contrast, the elevated LV
re created by aortic constriction
increase vascular compression and re-
lood flow. The observed elevation in
P/dt would also contribute to reduced
flow by a similar mechanism. In the
l LV, the majority of blood flow oc-
luring diastole when extravascular
essive forces are minimal (14). The
ardia which accompanied pressure
ad in rats would restrict the coronary
period by impinging upon the dia-
hase of the contractile cycle. Thus, in
ng the hemodynamic factors which
ice coronary blood flow, a reduction
rall coronary flow with LV pressure
ad would not be completely unex-
pected. Furthermore the ENDO portion of the LV is considered to be marginally perfused under normal circumstances (5, 6) and would be profoundly affected by even a minor reduction in coronary blood flow. Sympathomimetic agents have been shown to divert blood flow away from the ENDO portion of the LV (15). Taken together, the hemodynamic measurements obtained in the present study suggest that the cardiovascular system of the aortic constricted animals was operating under conditions of enhanced sympathetic stimulation. Increased sympathetic activity in aortic constricted animals would further reduce the already limited oxygen available to the ENDO portion of the hypertrophied LV.

Previous work by Myers and Honig (16) has shown that the ENDO portion of the normal LV possesses a greater vascular density than the EPI portion. In the present studies, a similar relationship was found in the ENDO-EPI alkaline phosphatase specific activities of control rat LV. Therefore, alkaline phosphatase specific activity would appear to be a valid chemical marker for myocardial vascular density. Concentric LV hypertrophy is created by pressure overload conditions. If concomitant vascular growth does not accompany ENDO tissue accumulation, then a decreased ENDO vascular density would result. Morphological studies (17, 18) and an evaluation of vascular capacity by a radioisotopic method (19) have shown a decreased vascular density in the LV of hypertrophied hearts. In the present studies it would appear that accumulated ENDO tissue outstrips its vascularity in the LV which has enlarged in response to a

pressure overload stimulus. In contrast, protein, nucleic acids and connective tissue increased in proportion to the increase in LV mass. The resulting loss of a preferential ENDO vascular density gradient could impose further flow limitations upon the normally, marginally perfused portion of the ventricle.

Since blood flow was not measured in the present studies, no conclusions can be drawn regarding the actual level of blood flow in the hypertrophied LV. Nevertheless, alterations in both hemodynamic factors and coronary vascular density were identified as being potentially capable of reducing blood flow to the hypertrophied LV, particularly to the ENDO portion.

Summary. LV pressure overload was created in adult male rats by abdominal aortic constriction. Three weeks following surgery, LV weight was increased by approximately 25% in aortic constricted animals. Aortic constriction produced a substantial pressure overload, as indicated by a 40 mmHg increase in peak LV pressure. Significant increases in heart rate and max dP/dt accompanied the pressure overload. The above hemodynamic factors would tend to reduce blood flow to the LV by (a) increasing vascular compression and (b) restricting the coronary inflow period. Alkaline phosphatase specific activity was utilized as an index of vascular density in the EPI and ENDO portions of control and hypertrophied LV. In control LV, the specific activity of the ENDO portion was significantly greater than the EPI portion, indicating a greater ENDO vascular density. The preferential ENDO vascular density gradient was eliminated in the LV which had enlarged in response to a pressure overload stimulus.

' THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 427-430 (1977)

ɔd Release of LH and FSH and Depletion of Pituitary Gonadotropin Content · Administration of [D-LEU⁶, desGly-$NH_2$¹⁰]-LH-RH Ethylamide¹ (39686)

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:orporation of D-amino acids in po- of the LH-RH decapeptide chain analog more potent and longer- ın the natural hormone (1). The tion of D-Leucine in position 6 as e substitution of the glycine amide :rminus with an ethylamide group, the LH and FSH releasing activi- more. Thus, [D-Leu⁶, desGly- H-RH ethylamide is 20-60 times ent than LH-RH (2). Recently, *al.* (3) have claimed that the super- alogues are merely more potent r parent hormone and lack pro- :tivity. In order to verify this as- , several doses of [D-Leu⁶, desGly- H-RH ethylamide were injected ıtterns of release of gonadotropins l with those produced by different ɔf LH-RH, using a sensitive imma- rat assay (2, 4, 5). In addition, as been demonstrated that injec- rge doses of purified porcine LH- uces a depletion of pituitary LH 6) and since there is also some vidence that a depletion of pitui- dotropin content may occur either olonged stimulation (7) or several with synthetic LH-RH (8), the [D-Leu⁶, desGly-$NH_2$¹⁰]-LH-RH le on the pituitary content of go- n were investigated as well.

ls and methods. Twenty-five-day- rats of the ARS Sprague-Dawley re used for all experiments. They ısed in quarters with controlled ıre (about 22°) and light (14 hr 10 hr darkness), and had free ac- ırina laboratory chow, fresh vege- tables, and water. For the immature male rat assay, groups of four animals each were injected with either synthetic LH-RH (50 and 150 ng/rat) or [D-Leu⁶, desGly-$NH_2$¹⁰]-LH-RH ethylamide (3, 9, and 50 ng/rat) or vehicle alone (0.2 ml of 0.1% gelatine/ 0.9% NaCl solution). Blood was collected by decapitation at different times after injections and the sera obtained by centrifugation was frozen until assayed for gonadotropins. Means of serum LH and FSH levels in every group were calculated, analyzed by Duncan's new multiple range test (9) and plotted on an arithmetic graph against time; the areas under the curves were arbitrarily taken to represent the integrated serum hormone levels (2, 4, 5). For the depletion test, the animals were injected with either 1 or 10 μg of [D-Leu⁶, desGly-$NH_2$¹⁰]-LH-RH ethylamide and blood and pituitaries were collected after 2, 4, 6 and 8 hr. The sera were separated by centrifugation. Anterior pituitaries were homogenized in 1 ml of 0.9% NaCl and frozen until assayed for gonadotropin content. In all experiments, LH and FSH were measured by radioimmunoassay using the methods described by Niswender *et al.* (10) and Daane and Parlow (11), respectively. NIH-LH-S17 and NIAMD-FSH-RPI were used as standard preparations. The details of these radioimmunoassays were described previously (7, 12). Hormone levels in pituitary depletion test were compared by Duncan's new multiple range test (9).

The LH-RH analog was prepared by the solid phase method as described previously (2).

Results. Figure 1 shows the patterns of gonadotropin release induced by various doses of LH-RH and [D-Leu⁶, desGly-$NH_2$¹⁰]-LH-RH ethylamide. It can be seen that, when LH-RH was given, serum levels

ork was supported by NICHHD Contract J.S.P.H.S. Grants AM 07467 and HD the Veterans Administration.

7 by the Society for Experimental Biology and Medicine

ISSN 0037-9

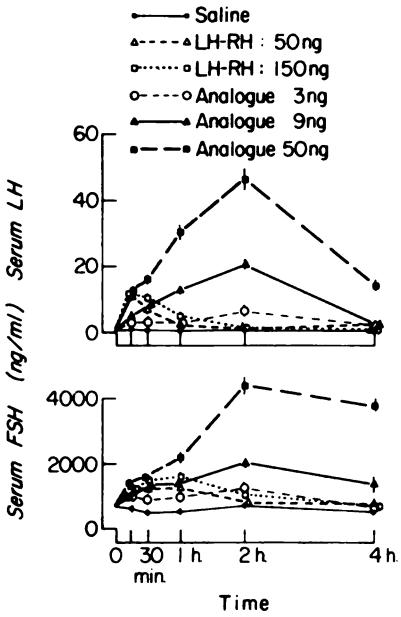

FIG. 1. Serum LH and FSH levels after sc administration of several doses of H-LH and [D-Leu^6, desGly-NH_2^{10}]-LH-RH ethylamide in immature male rats. Vertical lines indicate the standard error of the mean. Each point represents a mean of four rats.

of gonadotropin started to increase immediately after injections, reaching a peak at 15 min for LH and between 30 to 60 min for FSH. They then declined, reaching control levels at 2 hr. In comparison, the LH-RH analog, at all doses used, produced a completely different pattern of gonadotropin release, reaching a peak at 2 hr for both LH and FSH. At 4 hr, serum LH of the group treated with 50 ng of the analog remained elevated ($P < 0.05$ as compared to LH-RH treated group) as did the FSH serum levels of the groups treated with 50 ($P < 0.01$) and 9 ng ($P < 0.05$) of the analog.

Potencies of [D-Leu^6, desGly-NH_2^{10}]-LH-RH ethylamide calculated from the curves of Fig. 1 are presented in Tables I and II. The potencies varied according to the doses of the analog H and LH-RH used. The LH-releasing activity of the analog was 20 to 60 times greater (Table I) and the FSH-releasing activity was 10 to 30 times higher than that of LH-RH (Table II).

The effect of administration of 1 or 10 μg of [D-Leu^6, desGly-NH_2^{10}]-LH-RH ethyl*amide on serum LH* and FSH and pituitary content of gonadotropin is shown in Fig. 2. Serum LH after injection of both doses of analog remained elevated for up to 6 hr and FSH levels were still higher at 8 hr when

TABLE I. LH-RELEASING ACTIVITIES OF [D-LEU^6, desGly-NH_2^{10}]-LH-RH ETHYLAMIDE (ANALOG) BASED ON THE COMPARISON OF INTEGRATED SERUM LH LEVELS RELEASED BY THE ANALOG VS THOSE RELEASED BY LH-RH

Dose Analog (ng/rat)	LH-RH (ng/rat) LH-RH (ng/rat)	
	50	150
3	25.9	51.1
9	32.2	63.5
50	20.9	35.7

TABLE II. FSH-RELEASING ACTIVITIES OF [D-Leu^6, desGly-NH_2^{10}]-LH-RH ETHYLAMIDE (ANALOG) BASED ON THE COMPARISON OF INTEGRATED SERUM FSH LEVELS RELEASED BY THE ANALOG VS THOSE RELEASED BY LH-RH.

Dose Analog (ng/rat)	LH-RH (ng/rat)	
	50	150
3	17.3	33.6
9	15.0	31.3
50	9.5	18.6

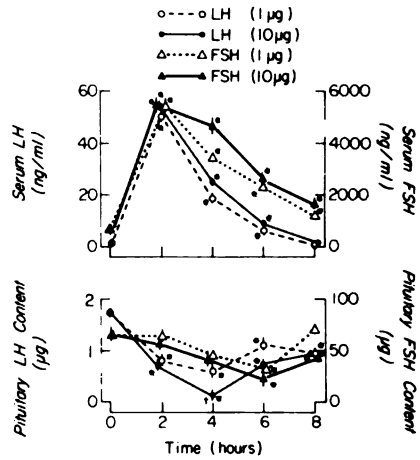

FIG. 2. Serum LH and FSH levels and gonadotropin pituitary content after sc injection of 1 or 10 μg of [D-Leu^6, desGly-NH_2^{10}]-LH-RH ethylamide in immature male rats. The analogue was injected at 0 time. Each point represents a mean of four rats. Vertical lines indicate the SEM. Duncan's new multiple range test: * Significantly different from the value of respective gonadotropin level at 0 time. +Significantly different from the value of the respective group injected with 1 μg of the analog.

vith basal values. The injection
μg of [D-Leu⁶, desGly-NH_2^{10}]-
ıylamide produced a significant
the pituitary content of gonado-
content was lower than the
ı value at 2 hr ($P < 0.05$) fell to
alue at 4 hr ($P < 0.05$), and was
basal levels at 8 hr ($P < 0.05$)
on of both doses. FSH content
ed to a lesser extent, reached a
r ($P < 0.05$) and was similar to
ı values at 8 hr in the group
ı 1 μg of analog.

n. Rippel *et al.* (3) have sug-
the superactive analog of LH-
ıted in positions 6 and 10 are
e active than their parent hor-
ɔut having prolonged activity.
d that [D-Leu⁶, desGly-NH_2^{10}]-
ıylamide produced a peak of LH
H-RH in diestrous rats, but the
ıe gonadotropin levels was simi-
peptides. They argued that this
nexplained delay in LH increase
ıjection of the analogue rather
tracted effect. In contrast, the
ented here show that in imma-
ıts even a small dose of [D-Leu⁶,
^{10}]-LH-RH ethylamide (3 ng/
ı peak at 2 hr (compared to 15
ı dose of LH-RH) and produces
y different pattern of gonadotro-
than that brought about by any
-RH. Furthermore, we have ob-
ame profile of LH release when
sGly-NH_2^{10}]-LH-RH ethylam-
is equal in potency to [D-Leu⁶,
^{10}]-LH-RH ethylamide[2] was
ɔ LH-RH using doses adjusted
es in potencies in diestrous I day
ıs II day rats (unpublished data).
ts our previous contention that
tive analogues of LH-RH ex-
ged effects (2).

and FSH releasing activities of
sGly-NH_2^{10}]-LH-RH ethylam-
epending on the doses of either
LH-RH given (Tables I and II).
be expected since nonparallel
relationships exist between the
se peptides and their responses.
he administration of relatively
f either LH-RH (100 ng/rat) or
[D-Leu⁶, desGly-NH_2^{10}]-LH-RH ethylamide (10 ng/rat) did not alter the pituitary content of gonadotropin (results not shown), the LH-RH analog given in doses of 1 or 10 μg/rat produced a depletion of the pituitary LH and FSH content (Fig. 2). The pituitary LH content fell to its lowest value at 4 hr after injection whereas the FSH content reached a nadir at 6 hr. The differences between the profiles are presumably due to different patterns of secretion of LH and FSH by the gonadotrophs (6). These results demonstrate that the gonadotropin pituitary content can be depleted by LH-RH analog. We reported previously that large doses of purified porcine LH-RH also induced some depletion of pituitary LH-content in rats (6). These observations should be taken into account when administering repeatedly or infusing continuously LH-RH or its analog to humans (8) and rats (7).

Summary. LH and FSH releasing activities of [D-Leu⁶, desGly-NH_2^{10}]-LH-RH ethylamide were examined using immature male rats. The gonadotropin release induced by various doses of [D-Leu⁶, desGly-NH_2^{10}]-LH-RH ethylamide followed a different pattern than when LH-RH was given, reaching a peak at 2 hr after injections, serum LH and FSH being still elevated at 4 hr. In contrast the LH and FSH release induced by LH-RH reached a peak in 15 min and declined at 1 hr. This provides further evidence that this analog is indeed long-acting. The LH-RH analog had a potency 20-60 times greater for LH release and 10-30 times higher for FSH release than LH-RH. [D-Leu⁶, desGly-NH_2^{10}]-LH-RH ethylamide also caused a significant depletion of the pituitary gonadotropin content.

The authors express their thanks to Mrs. J. Gauthier and Mrs. D. Pierson for their technical help, and to Dr. G. Niswender, Dr. A. Ward and NIAMDD Rat Pituitary Program for the gift of the Material used for radioimmunoassays.

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Received September 20, 1976. P.S.E.B.M. 197 154.

ıGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 431-434 (1977)

tro Suppression of *Herpesvirus saimiri* Replication by Phosphonoacetic Acid[1] (39687)

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ɔsphonoacetic acid (PAA) has been ı to inhibit the replication of several ɔers of the herpesvirus group in model ıl systems as well as in tissue culture . Several of these studies (2, 3, 6) have nstrated that PAA exerts the effect by ically inhibiting the virus induced polymerase. The present report is rned with the *in vitro* effect of PAA on plication of the oncogenic simian her- us, *Herpesvirus saimiri* (HVS).

terials and methods. Cells, virus and its. Two continuous lines of owl monidney (OMK) cells established in our ıtory, OMK 637-69 and OMK 210- ere used. These cells were grown and ained following standard procedures HVS stock E940-F and *Herpesvirus is* HVH type 1, stock E-97-M pre- in OMK cells were used in these studiters were calculated by the method of and Muench (8). PAA was kindly ded by Abbott Laboratories, North go, Ill.

A treatment. Unless otherwise stated, ed cells were incubated for 1 hr for adsorption, and treated with 100 μg/ PAA and then observed for 15 days.

:tron microscopy and staining proce- Techniques to process infected OMK or electron microscopic (EM) exami- ı have been described previously (9). ıethods for staining infected cells with :oxylin and eosin (HE) and the immunofluorescent antibody (IFA) test are standard procedures described elsewhere (10).

Results. Effect of PAA on HVS and Herpesvirus hominis type 1 (HVH). PAA at a concentration of 100 μg/ml was found to reduce the infectivity of HVH by 2.0 to 3.0 logs and that of HVS by over 5.0 logs which was a total inhibition of infectivity.

Determination of minimum effect doses of PAA to inhibit HVS replication and effect of drug on OMK cells. Groups of cultures infected with approximately 100 and 1000 $TCID_{50}$ of HVS were treated with PAA at varying concentrations as indicated in Table I and then observed for 15 days. Concentrations of 1–20 μg/ml had no effect on HVS replication. Forty μg/ml of PAA partially inhibited both concentrations of HVS and 60 μg/ml stopped virus replication.

The effect of variable concentration of PAA on fully grown OMK monolayers was also studied. For this, groups of OMK 637-69 cultures were treated with PAA doses of 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mg/ml. Cultures treated with up to 1 mg/ml remained apparently normal during the 15 days of observation. Higher concentrations caused rounding of cells with subsequent degeneration. OMK cells maintained on 100 μg/ml of PAA for 15 days were sequentially transferred in media containing the drug. They grew to confluency for two additional passages and thereafter slowed down and appeared affected by the drug. However, if growth media without PAA was fed to the cells they returned to their normal morphology and growth pattern.

Effect of PAA on HVS replication. As observed in preliminary experiments, PAA in effective and nontoxic concentrations completely inhibited HVS production (Table II). The compound had no effect on HVS replication when virus was treated

s study was conducted under Contract Nos. P 33390 and NCI-NO1 CP 43225 within the ancer Program of the National Cancer Institute, HS.

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ISSN 0037-977

with PAA for 24 hr and held at 4° before the adsorption period. Pretreatment of OMK cells for 24 hr before the addition of virus had no effect on HVS replication.

A representative growth curve of HVS in untreated and PAA treated cells, is shown in Fig. 1. In this experiment a group of 35 mm Falcon dishes containing confluent monolayers of OMK cells were infected at an input multiplicity of approximately 0.01/ per cell; after adsorption for 1 hr, the cultures were fed with fresh media and two cultures (cells plus media) were harvested every 24 hr to test for total virus. Seventy-two hours after infection, the normal maintenance media was replaced with PAA containing media in half the dishes, and parallel harvesting procedures were followed for an additional 15 days. Figure 1 shows that HVS replication in the untreated cells followed a predictable pattern. Maximum virus production with a CPE of three (approximately 75% of the cells involved) was

TABLE I. MINIMUM EFFECTIVE CONCENTRATION OF PAA TO CONTROL REPLICATION OF HVS IN OMK CELLS.

Concentration of PAA (μg/ml)	Degree of CPE HVS Concentration ($TCID_{50}$)	
	100	1000
200	0[a]	0
100	0	0
80	0	0
60	0	0
40	1	2
20	4	4
10	4	4
1	4	4
0	4	4

[a] = degree of CPE at day 15.

TABLE II. EFFECT OF PAA ON THE REPLICATION OF HVS IN OMK CELLS.

Treatment	Virus titer (log_{50} $TCID_{50}$/0.1 ml)		
	Control	PAA (100 μg/ml)	Reduction in titer
I. PAA treatment of infected cells for 15 days	5.5	0.0	5.5
II. Pretreatment of cells with PAA (24 hr)	5.0	5.0	0.0
III. Pretreatment of virus with PAA (24 hr)	4.0	4.5	0.0

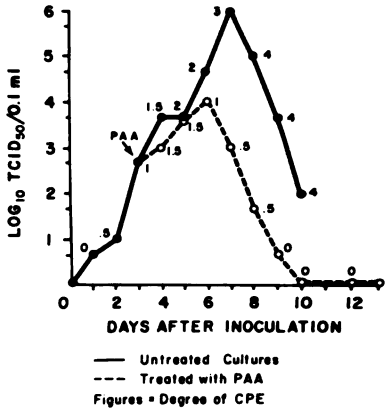

FIG. 1. Growth curve of HVS in PAA treated and untreated OMK cells. PAA was added 72 hr after the infection of the cells. Titer in untreated cultures ●——●, PAA treated cultures O- - -O, and degree of CPE (figures).

reached in 7 days and then the titer of HVS dropped steadily as expected.

The treated cells had a CPE of 1 and the titer was $10^{2.5}$/0.1 ml at the time when the PAA was added (72 hr). The increase in CPE and virus titers proceeded to a maximum of 1.5 and $10^{4.0}$/0.1 ml respectively in the next 2-3 days, then dropped steadily until reaching 0 between 9 to 12 days after infection. At this point the monolayers looked apparently normal and "cured" of HVS infection.

Effect of PAA on HVS replication when added at varying times. A group of OMK cells were infected with 1000 $TCID_{50}$ of HVS. PAA was added to these cultures at times 0, 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, 96, 120 and 144 hr postinfection. There were groups of five 35mm dishes for each time period. The cultures were kept until they were destroyed by the virus or observed for 15 days after the infected cultures recovered from CPE. Addition of PAA from 0 to 24 hr after virus adsorption resulted in no CPE. No virions, antigens or inclusions could be detected in these cells. Cocultivation of these cells with OMK cells did not result in recovery of infectious virus.

Recognizable CPE could be seen in cultures between 24 and 48 hr after infection. If PAA was added at this time the CPE disappeared. If these cultures were held in PAA containing media for 15 days and then

replaced with growth media without PAA, the CPE would reappear again. The infected cultures showing CPE involving a little more than 50% of the monolayer (CPE reading 2.5) recovered from CPE if PAA was added up to 120 hr after infection. Addition of PAA at 144 hr postinfection did not arrest further spread of infection and the monolayer was destroyed (Table III).

Long term persistence of HVS in OMK infected cells treated with PAA. A series of OMK monolayers were inoculated with 1000 TCID of HVS and maintained on media containing PAA, for a total of 63 days. During this time, at 7-day intervals the PAA media was replaced with normal growth media from two dishes and observed for the development of CPE. All these cultures developed CPE. The only difference observed was an increase in the lag period required for the appearance of CPE in cultures taken on days 7, 14, 21 and 28. However, from this point on, CPE appeared always between 19 and 23 days after withdrawal of PAA media.

Discussion. The oncogenicity of HVS in several species of monkeys and rabbits has been well established by several investigators. Our purpose in this study was to test the compound PAA on a known oncogenic virus, so as to conduct therapeutic trials in animal models if the compound was found to be effective in the *in vitro* system.

The results of our study presented in this report clearly shows that PAA inhibits the replication of HVS at a concentration of 60 μg/ml and above. Other investigators have shown that the replication of many herpesviruses are affected by PAA (1-6). To this growing list HVS may be added.

We have not looked into the mechanism of the action of PAA on HVS. In a very recent report PAA was found to affect the DNA polymerase of HVS (6). We found that pretreatment of cells or virus had no inhibitory effect on HVS replication. This would indicate that PAA did not affect virus adsorption or penetration of HVS in OMK cells. It also indicated that PAA did not affect the intact HVS.

Long term maintenance of PAA (63 days) on infected cells also had no effect on completely suppressing replication of HVS. Withdrawal of PAA after this period resulted in virus replication and destruction of infected monolayers. The presence of PAA

TABLE III. EFFECT OF ADDITION OF PAA AT DIFFERENT TIMES AFTER ADSORPTION OF *Herpesvirus saimiri*.[a]

Time for addition of PAA (hr)	Cytopathic effect: Degree at PAA addition time	Cytopathic effect: Maximum CPE reached	Days to recover from CPE	Appearance in recovered cells withdrawn from PAA[d]	HVS detection in recovered cells[e]
Control		4.0[b]			+
0	0.0	0.0			−
1	0.0	0.0			−
2	0.0	0.0			−
3	0.0	0.0			−
4	0.0	0.0			−
6	0.0	0.0			−
9	0.0	0.0			−
12	0.0	0.0			−
24	0.0	0.5	3[c]	+	−
48	0.5	1.0	5	+	−
72	1.0	1.5	6	+	−
96	1.5	2.0	6	+	−
120	2.0	2.5	9	+	−
144	2.5	4.0			

[a] = infected with 10^3 $TCID_{50}$.
[b] = 100% destruction of monolayer.
[c] = days after PAA addition.
[d] = withdrawn 15 days after recovery.
[e] = tested by infecting OMK cells, electron microscopy and staining procedures for antigens by immunofluorescence and inclusion by HE staining.

permitted the virus to remain in a quiescent state which became reactivated on removal of the drug. Thus PAA does not have a virucidal effect on HVS. For its effectiveness to be felt, its continued presence appears to be necessary.

PAA has the remarkable property of being effective when added to HVS infected cultures even 120 hr postinfection. This was at a time when a little over 50% of the monolayer showed cytopathic effect. Addition of PAA at this point effected a "cure" and the cells appeared to have recovered. This shows that PAA was able to prevent further replication of the virus after the initial replication that took place during the 120 hr before the addition of PAA. This is somewhat similar to the effect of PAA when applied as an ointment to rabbit keratitis and mice dermatitis (1). However, in the tissue culture system removal of PAA from infected but apparently "cured" cell cultures results in reappearance of CPE.

Summary. Replication of *Herpesvirus saimiri* was inhibited by 60 μg/ml of phosphonoacetic acid (PAA) in owl monkey kidney cell cultures (OMK). OMK cells were not adversely affected by PAA. Concentrations over 1 mg/ml of PAA proved toxic to OMK cells. Pretreatment of virus or cells prior to infection had no inhibitory effect on HVS. The continued presence of PAA was required to suppress HVS replication. Re-

ıGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 435-438 (1977)

Effects of Morphine Pellet Implantation in Neonatal Rats (39688)

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: newborn rat has been used success- to study long-term behavioral and pi- y-adrenal effects of early exposure to tics (1, 2). Since early handling is n to exert profound influences on the opment of behavioral and neuroendo- functions in the rat (3), the effect of injections and of repeated handling ıave obscured drug-induced changes in earlier studies. To minimize the influ- of manipulation (while avoiding prob- ıssociated with direct administration of ·ug to the mother), morphine (M) was ıistered to rat pups by pellet implanta- This report describes developmental ısting effects of this standardized treat- at different times during early postna- :.

terials and methods. The pellets used prepared according to the method of ın and Tingstad (4). Each pellet con- l 75 mg of morphine base. Placebo s (P) were prepared using the same t of lactose.

ial studies showed that subcutaneous ntation of one M pellet in 8-day-old resulted in death within a few hours, all animals implanted with P pellets ·ed. In subsequent work, pellets were d with beeswax and scored on each vith a needle: one scratch for animals · 1 week of age and two scratches for animals. All pellets were implanted taneously in the flank using brief ether ıesia.

study long-lasting effects of early M implantation, newborn Sprague- ey female rats (Simonsen Farms, Gil- Calif.) were placed in litters of eight naintained under standardized condi- of lighting (14-hr illumination alternat- ith 10-hr darkness) and temperature : 1°). Two pups from each litter re- l either one M or one P pellet on Day l1 of postnatal life. These times were n to coincide *with times* of daily injections of M in earlier studies (1, 2). Mortality was noted and surviving pups were subsequently handled only briefly at weekly intervals to measure body weight. All animals were weaned at 21 days of age and housed in pairs thereafter.

The effects of early implantation of M on resting pituitary-adrenal function were studied at 1600 hr on Day 43. Each animal was subjected to ether anesthesia and removal of a blood sample by jugular venipuncture as described previously (5). The plasma was separated and frozen and used subsequently for fluorometric determinations of corticosterone concentrations (6).

To determine whether early pellet implantation produced long-lasting tolerance to M, the analgesic response to a challenge dose of M was examined on Day 48 using the hot-plate test (7). After three control trials, in which response latencies (time to raise and lick a paw) were measured at 30-min intervals, each animal received a subcutaneous injection of M (10 mg/kg). Thirty minutes later each animal's response latency was again obtained. The mean control latency was calculated for each animal and subtracted from the value obtained 30 min after M injection to determine the change in response latency.

On Day 68, tolerance to M was again tested by challenging animals from each treatment group with a subcutaneous injection of 40 mg/kg of M. Thirty minutes later a blood sample was obtained by etherization and jugular venipuncture for determination of the plasma corticosterone response.

Since decreased survival of the offspring of female rats treated with M prior to mating has been reported (8, 9), a final test was performed to study fertility, litter size, and offspring mortality of rats addicted to M early in life. Five animals from each treatment group were bred with proven sires at approximately 120 days of age.

Statistical analyses were performed using

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ISSN 0037-9

Student's t test. A probability of 0.05 or less was considered significant.

Results. Mortality rates were 36 or 25% for animals implanted with M on Day 5 or 11, respectively, while only one P-implanted animal died (4%). The majority of deaths occurred within 24 hr after M pellet implantation, 64 and 52% of deaths among Day-5 and Day-11 animals, respectively.

As shown in Fig. 1, survivors of both M treatment groups showed immediate suppression of body weight gain compared to P-implanted controls, and these deficits remained significant ($P < 0.05$) throughout the 126 days of the experiment. Body weight depressions of the Day-5 M group appeared to occur more gradually than those of the Day-11 M group. Differences between the mean weights of the Day-5 M and P groups were 6.7 g on Day 12 ($P < 0.01$) and reached a maximum difference of 23.5 g on Day 63 ($P < 0.01$). Comparable differences in the Day-11 animals were 7.6 g ($P < 0.005$) on Day 12 and reached a maximum of 23.5 g ($P < 0.005$). Day-5 M-

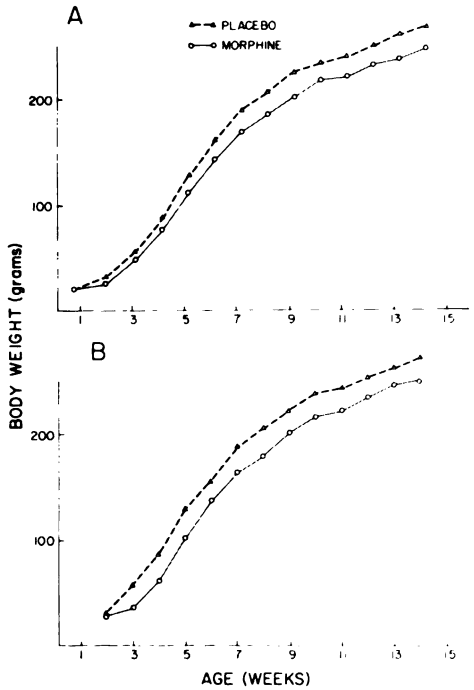

FIG. 1. Ponderal growth of female rats following neonatal implantation of morphine (○) or placebo (△) pellets at 5 (A) or 11 (B) days of age. Each point *represents the mean* weight of 10–12 rats.

e to the M challenge (10), and those of
l-treated groups did not differ signifi-

he breeding experiment, 19 of the 20
ıls became pregnant, the exception
one of the Day-5 M-treated animals.
sizes of the four treatment groups
d from 6 to 12; mean numbers of pups
9.3, 9.8, 10.0, and 10.0 for Day-5 and
and P groups, respectively, and mor-
rates in these same groups were 61,
7, and 52% by postnatal Day 11. It
ecessary to terminate the experiment
ı time, so the ability of the mothers to
ain survival of the pups for a longer
ı remains uncertain. Although the
lity rates in all groups were quite high,
results show that female rats exposed
ɔnatal implantation of M or P, as de-
d in this experiment, can breed suc-
lly and produce normal size litters.

cussion. The results of these studies
nstrate the feasibility of producing in
ɔng-lasting suppression of growth and
ıased sensitivity to M by implantation
ngle M pellet early in life. The mortal-
ɔ–36%) associated with this method of
ministration is comparable to that re-
ʒ from daily injections of M during the
ɔr second week of postnatal life (2).
acts that cyanosis and hypothermia
observed in M-treated animals to-
with the low mortality rate of the P-
d groups, indicate that hypoxia due to
luced respiratory depression, rather
ether anesthesia and surgery, was an
tant cause of death. Hypoxia also
have contributed to the protracted
s observed following neonatal M-im-
ıtion.

persistent reduction of body weight
ved following M pellet implantation
bles the somatic response of rats given
ections during the prenatal (9, 11, 12)
ly postnatal periods (1, 2). We have
ved similar suppression of body weight
ʒ at least 16 weeks in female rats im-
d with a morphine pellet at 14 days of
unpublished observation). The fact
similar ponderal response follows M
ıistration over a wide range of devel-
ıtal stages suggests that a lack of criti-
ıing is required for producing this opi-
ate effect. It is not clear, however, that the same processes are responsible for weight depressions in the two M groups. Weights of Day-5 M-treated animals were depressed more gradually, reaching maximum depression on Day 63, than those of Day-11 M-treated rats, which reached a maximum depression 14 days after the implantation. This same general pattern of slower versus faster rates of body weight depression was observed in animals injected with M during the early (Days 3–12) vs the late (Days 12–21) preweaning period. The greater mortality of the Day-5 animals suggests caution in the interpretation of comparative differences among survivors and suggests that the younger animals may have suffered greater damage due to hypoxia. This possibility is consistent with the greater degree of cyanosis observed in the younger animals following M implantation. It is noteworthy that Bass and Lundborg (13) found greater depression of respiratory rates in younger (Day 5) compared to older (Day 30) rats.

In this as in previous injection studies (1, 2), rats showing M-induced suppression of body weight failed to evidence catch-up growth following cessation of drug administration. The reason for this is not clear but may relate to the duration of neonatal drug exposure since we have observed increased weight gain in growth-retarded rat pups following cessation of M injections for just a few days (unpublished observation). Growth inhibition appears to be a common effect of opiates since it has been observed in other rodents (14) and in human infants born to opiate-addicted mothers (15).

Tolerance to the analgesic action of M observed on Day 48 in animals implanted with M on Day 11 is consistent with results obtained previously (1, 2). Another advantage of the pellet implantation method is that it may not subject the pup to the stress of withdrawal. Since the stress of withdrawal may have lasting effects, it must be considered in studies wherein daily injections are terminated. Pellets implanted in this study were not removed, which possibly led to a gradual decrease in the amount of M released over a period of time. Although signs of M withdrawal were not seen in any rats during the periods of observation in this

study, verification of a lack of withdrawal effects will require additional study. Finally, it should be mentioned that pellet implantation is a rapid and convenient way to administer M. The present study showed that this method, like daily injections of M, is capable of producing protracted suppression of growth and sensitivity to M in rats.

Summary. A morphine pellet implantation method has been modified for use in very young rats. Compared to P-implanted controls, implantation of an M pellet on either Day 5 or 11 results in diminished body weight for periods at least up to 15 weeks. Such treatment on Day 11 is associated with reduced sensitivity to the analgesic action of morphine (10 mg/kg) when tested on Day 48 using the hot-plate technique. Resting pituitary–adrenal activity in animals implanted with morphine on Day 5 or 11 was within normal limits when examined on Day 43. Corticosterone responses to a challenge dose of morphine (40 mg/kg) on Day 68 failed to demonstrate tolerance unequivocally. These findings are consistent with those obtained earlier with daily injections of morphine and demonstrate that the long-lasting ponderal effect and tolerance to the analgesic actions of morphine may be produced without the confounding effects of daily handling and stress of abrupt withdrawal in neonatally addicted rats. This convenient method of prolonged early morphine exposure in young rats may be used as an experimental model for the study of long-range effects of neonatal exposure to narcotics.

We wish to thank Mr. Andrew Baldwin for his

: SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 439–443 (1977)

ic Assistance of 5-Iodo-2'-Deoxyuridine Penetration into Neonatal Mouse Skin and Effects on DNA Synthesis[1, 2] (39689)

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deoxyuridine (Idoxuridine, nalog of thymidine, inhibits the of mammalian cells, bacteria, ontaining viruses (1–3). IdUrd by Kaufman to be effective in lex eye infection (4), and the ical therapy of herpes simplex IdUrd has been verified (5–7). he usefulness of this drug in gy has led to its acceptance as agent. Some reports have IdUrd is effective in animals py of cutaneous lesions caused nplex virus (8, 9), but another ed lack of efficacy in skin le- the poor transport of the com- eous solution through skin bar- Urd in an adherent vehicle was r herpes simplex labialis with sults (11), again suggesting tion.

phoresis is a method for assur- on of charged drugs into sur- this method might overcome of IdUrd penetration into skin. s has been used in medical and to increase the penetration of 3). The experiments presented erformed to observe the effect esis on penetration of IdUrd l mouse skin and to determine iontophoresis of IdUrd upon dR) metabolism in this rapidly tissue.

nd methods. Animals. Albino -Webster-NCI, outbred, neo- to 3 g body weight and 2–3 vere used.

Radioactive materials and chemical. [^{125}I]IdUrd was purchased from New England Nuclear Co. (sp act, >200 Ci/mmole). [^{3}H]TdR was obtained from Schwarz/Mann Bioresearch (sp act, 15.6 Ci/mmole). IdUrd in mannitol (50:50) was kindly supplied by Calbiochem and, when it was dissolved in demineralized-redistilled water at 0.125%, the solution had a pH of 9.2, osmolarity of 10 mosm, and Na content of 2.5 mequiv/liter.

Iontophoresis. The trunk of the mouse was wrapped with a cloth saturated with 0.3 ml of 0.125% IdUrd solution for topical application. For iontophoresis of IdUrd, a strip of aluminum foil was wrapped around the cloth and connected to an appropriate electrode, while the return electrode was connected to the tail of the mouse. The amount of current applied was 0.5 mA for 10 min (EMF = 11 V). To observe the IdUrd penetration into skin (Series I experiment), 0.125% IdUrd solution containing 5.0 μCi of [^{125}I]IdUrd/0.3 ml was applied to the mouse. Mice were divided into four groups as follows: Group A: topical application of IdUrd; Group B: anodal(+) iontophoresis of IdUrd (anode was connected to the aluminum foil); Group C: cathodal(−) iontophoresis of IdUrd (cathode was connected to the aluminum foil); Group D: injection of IdUrd (375 μg of IdUrd containing 5 μCi of [^{125}I]IdUrd was injected ip). Each group included eight mice and was divided into two subgroups in order to obtain two periods (15 and 300 min) after IdUrd administration. After drug administration, the mouse's skin was thoroughly washed. At the end of these times, the mice were sacrificed. A second series of experiments (Series II) was performed to observe the effect of IdUrd iontophoresis on TdR metabolism in skin. Each mouse was injected ip with 1.0 μCi of [^{3}H]TdR at 10 min after IdUrd administration. Series II mice

in part by NSF Grants (to JMH), No. S-73-01146, and BMS-75-16768.

reports were presented at the meeting : Academy of Sciences, 3rd Conference bstances, February 1976, and Internaon for Dental Research Annual Meetlorida, March 1976.

he Society for Experimental Biology and Medicine

ISSN 0037-9

were divided into five groups: Group E: control (no IdUrd administration); Group F: topical application of IdUrd; Group G: anodal(+) iontophoresis of IdUrd; Group H: injection of IdUrd (375 μg of IdUrd was injected ip); Group I: electrical control (anodal(+) iontophoresis of saline solution). Each group except group I included 12 mice and was divided into three subgroups to obtain three periods (15, 30, and 300 min) after [^{3}H]TdR injection. The mice were sacrificed at the end of these times. Group I contained only four mice sacrificed at 300 min.

Homogenates. After sacrifice, the skin of the trunk was removed, weighed and homogenized in 10 ml of 5% citric acid–8% sucrose solution per gram wet weight of tissue and processed according to the methods described by Hill *et al.* (19). Briefly, an aliquot of homogenate was mixed with 0.5 *N* $HClO_4$, and this mixture was centrifuged. The supernatant was designated as first acid-soluble fraction. This fraction was neutralized with 4 *N* KOH before separation of metabolites.

Separation of IdUrd, I^-, TdR, and thymidine monophosphate (TMP). IdUrd, TdR, and TMP were separated by thin-layer chromatography from the first acid-soluble fraction using a modification of the method described by Garrett *et al.* (20). Free I^- was separated from the acid soluble fractions with methyl mercuric bromide and toluene (21). The ^{125}I radioactivity of IdUrd or I^- was determined in a Beckman gamma counter, and the ^{3}H radioactivity of TdR and TMP was determined using a Beckman liquid scintillation counter.

Radioactivity of DNA. After washing with

TABLE I. RADIOACTIVITY OF [^{125}I]IdUrd AND FR ADMINISTRATION OF [^{125}I]IdI

Method of application	Radioactiv		
	[^{125}I]IdUrd		
	(cpm)	(Δ)	
Topical	60 ± 9		2
Iontophoresis (+)	171[b] ± 23	+185%	1
Iontophoresis (−)	128[b] ± 16	+113%	8
Injection (ip)	685[b] ± 93	+1042%	28

[a] Each determinant contains data from four mice. Ski

[b] Significantly different ($P < 0.05$) from topical applica

fraction before thin-layer chromatography (tlc).

ıe drug by this method, whereas the · methods delivered only a fraction of ose.

ble II shows the incorporation of IdUrd into neonatal mouse skin DNA. 5 min after [^{125}I]IdUrd administration, cathodal(−) and anodal(+) iontopho- significantly increased the incorpora- of [^{125}I]IdUrd into DNA (621 and %, respectively) compared to topical cation. Also at 300 min, [^{125}I]IdUrd poration into DNA was significantly ased by cathodal and anodal iontopho- (109 and 69%, respectively).

ble III shows the effects of various ; of IdUrd treatment on TdR metabo- 300 min after IdUrd administration. radioactivity of [^{3}H]TdR in the skin soluble fraction was significantly in- ed in the groups pretreated with IdUrd ıphoresis or ip injection compared to r control or topical application, eas iontophoresis of saline did not alter adioactivity of [^{3}H]TdR compared to ntreated group. On the other hand, the radioactivity of [^{3}H]TMP was significantly decreased by IdUrd. These results indicate the inhibitory effect of IdUrd on thymidine kinase. Iontophoresis itself (saline control) did not alter [^{3}H]TMP radioactivity compared to the untreated group. At 15 or 30 min after IdUrd administration (data not shown), there were no significant differences between iontophoresis and the topical application group.

In Fig. 1, the incorporation of tritiated thymidine into neonatal mouse skin DNA is indicated. The control group (top line), which received only [^{3}H]TdR, exhibited a linear increase in specific activity of DNA with time. The mice treated with IdUrd topically had an initial depression of incorporation at 15 min, with slight depression at 30 min, and recovery almost to the level of the control at 300 min. The mice treated by iontophoresis of IdUrd exhibited depressed incorporation of TdR into DNA, resembling that seen in the ip treated group (bottom line) especially at the 300-min time. The same inhibition was noted for the ionto-

TABLE II. INCORPORATION OF [^{125}I]IdUrd INTO NEONATAL MOUSE SKIN DNA.[a]

hod of application	Radioactivity (cpm/mg of DNA ± SE)			
	Labeling time			
	15 min		300 min	
	(cpm)	(Δ)	(cpm)	(Δ)
ıl	216 ± 35		763 ± 121	
ıhoresis (+)	833[b] ± 116	+286%	1,290[b] ± 123	+69%
ıhoresis (−)	1,588[b] ± 408	+621%	1,596[b] ± 198	+109%
on (ip)	12,863[b] ± 1823	+5855%	69,199[b] ± 855	+8969%

ach determinant contains data from four mice.
gnificantly different ($P < 0.05$) from topical application.

ABLE III. EFFECT OF IdUrd ON [^{3}H]TdR AND [^{3}H]TMP RADIOACTIVITIES IN SKIN ACID-SOLUBLE FRACTION OF NEONATAL MICE.[a]

ethod of IdUrd application	Radioactivity (dpm/mg of wet tissue ± SE)			
	[^{3}H]TdR		[^{3}H]TMP	
	(dpm)	(Δ)	(dpm)	(Δ)
ıtreated controls	175 ± 5		110 ± 5	
pical	230[b] ± 5	+31%	65[b] ± 5	−41%
ıtophoresis(+)	425[b,c] ± 5	+143%	45[b] ± 10	−59%
Injection (ip)	870[b,c] ± 5	+397%	40[b,c] ± 5	−65%
ıtophoresis(+) of saline	180 ± 5	+3%	120 ± 5	+9%

ach determinant contains data from four mice. Skin samples were taken 300 min after application.
gnificantly different ($P < 0.05$) from untreated group.
ignificantly different ($P < 0.05$) from topical application group.

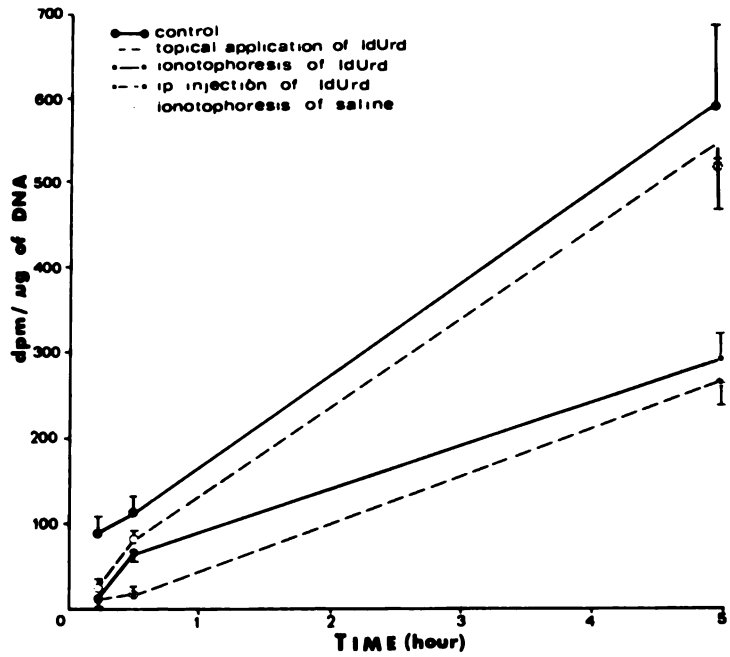

FIG. 1. DNA specific radioactivity of [^{3}H]thymidine in neonatal mice skin after application of IdUrd.

phoresis and the ip group at 300 min when we repeated the experiment (data not shown).

Discussion. The medical and dental literatures indicated that electrically charged drugs can be most effectively introduced into surface tissues by means of iontophoresis. Iontophoresis is used in the pilocarpine diagnostic test for cystic fibrosis (12) and for administration of vasodilators for peripheral vascular disease (13). Comeau *et al.* (14) reported local anesthesia of the ear by iontophoresis. Gangarosa (15) also reported successful local anesthesia of oral mucosa with lidocaine and epinephrine iontophoresis. Furthermore, fluoride iontophoresis appears effective for the treatment of exposed hypersensitive dentin (16–18). Based on these reports, the authors decided to study whether the antiviral drug, IdUrd, could be assisted in its penetration by iontophoresis. Although IdUrd does not possess an obvious ionic site, the replacement of the methyl group in position 5 of TdR with iodide alters the electron configuration of the pyrimidine moiety because of possible inductive and resonance effects of the halogen (3) which might result in the formation *of polar moieties in* solution. *In vitro* conductivity studies performed by us indicate that the IdUrd:mannitol solution has a low conductivity which is acceptable for application by iontophoresis (23).

The results of iontophoresis of IdUrd in mice indicate that penetration of IdUrd into skin can be increased by this method. As shown in the pharmacokinetic study of [^{125}I]IdUrd penetration into skin, cathodal or anodal iontophoresis effectively increased the penetration of IdUrd into neonatal mouse skin. Theoretically, in iontophoretic application, only one electrode should assist the penetration of an ionic drug, i.e., the penetration of negative ions will be assisted by cathodal iontophoresis while positive ions will be assisted by anodal iontophoresis. Since both cathodal and anodal iontophoresis assist IdUrd penetration, we believe that electro-osmosis (24) might be involved as an important factor in the penetration of IdUrd into skin. Preliminary results in our laboratory (data not shown) support the electro-osmotic hypothesis; we found that nonionic substances, in NaCl solution, can be assisted in their penetration by either cathodal or anodal iontophoresis. Also our IdUrd:mannitol solutions contained a small amount of sodium ion (see

ods section).

ıe mechanism of action of IdUrd on
\ viruses involves competitive antago-
of TdR to block the metabolic steps for
incorporation into DNA (1-3). The
ies presented here indicate that ionto-
esis of IdUrd may inhibit TdR phos-
ylation and incorporation into DNA as
tively as ip injection of IdUrd. This
lt suggests that the IdUrd may block
metabolism including incorporation of
ıidine triphosphate into DNA.

mmary. These experiments indicate
iontophoresis of IdUrd into neonatal
se skin may be as effective as ip admin-
tion in blocking TdR metabolism in-
ng its incorporation into DNA of the
Also, IdUrd appeared to be incorpo-
l into DNA. These results suggest that
iral chemotherapy in surface tissues
be better accomplished by using ionto-
etic assistance of penetration since the
is delivered specifically at the desired
and the overall dose to the animal is
l compared to systemic administration.
would expect that the drug which is
inistered by iontophoresis would proba-
ıct similarly on active virus-producing
to prevent viral multiplication.

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Received July 19, 1976. P.S.E.B.M. 1977, Vol. 154.

Role of Bovine Serum Albumin in Blast Phytohemaggl

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Human peripheral lymphocytes are activated to undergo blast transformation by specific and nonspecific stimulants *in vitro* (1–3). Supplementation with autologous serum or fetal calf serum to the medium is generally required for the transformation of lymphocytes. However, the factor(s) in serum which cause blast transformation of the stimulated lymphocytes are still unknown. The study of such factors in serum may reveal the mechanism of activation and proliferation of the stimulated lymphocytes. Polet and Spieker-Polet and others demonstrated that serum albumin itself was indispensable to the medium component for the blast transformation of lymphocytes by mitogens (4–6).

Recently, Yamane *et al.* have formulated a serum-free culture medium (SF medium) in which various mammalian tumor cells including a human lymphoid cell type can grow well (7). With this SF medium, albumin-bound fatty acids were found to play an important role in cell culture. Therefore, we undertook to investigate mitogen-induced transformation of human lymphocytes in this medium to see whether the albumin-bound fatty acids play a significant role in the transformation of lymphocytes. As a result, an important role of fatty acids in the phytohemagglutinin (PHA)-stimulated transformation of the lymphocytes was established.

Materials and methods. Basal medium and BSA. As described previously by Yamane (7) for the preparation of basal SF medium, Eagle's minimum essential medium (MEM) was supplemented with sodium pyruvate, insulin, putrescine, zinc sulfate, and various nucleosides. All components were dissolved in deionized distilled water and then sterilized through Millipore membrane filters. BSA (Cohn Fr.V) and defatted preparations of BSA were purchased from Armour

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trichloroacetic acid (TCA) were added to each tube. After a 2-hr incubation at 4°, the tubes were centrifuged, and resulting precipitates were washed twice with 5% TCA. The precipitates were dissolved in ethanol and then centrifuged. The pellets were hydrolyzed with 0.2 ml of 2 *N* sodium hydroxide at 80° for 2 min, and one drop of 4% $SnCl_2 \cdot 2H_2O$ was added. The samples were dissolved in 0.6 ml of Biosolv II (Beckman) and 10 ml of scintillation fluid (2,5-diphenyloxazole, PPO, and 1,4-bis[2-(5-phenyloxazolyl)]-benzene, POPOP) was added. The solution was counted in a Packard scintillation counter. The resulting counts were corrected to values of disintegration per minute (dpm) by referring to a quench curve established for the machine. Standard deviations were calculated from the values of triplicate tubes. The stimulation index was calculated with the following formula:

$$\text{Stimulation index (SI)} = \frac{\text{dpm of stimulated cells}}{\text{dpm of unstimulated cells.}}$$

Results. Optimal concentrations of BSA in SF medium. To determine the optimal concentrations of supplementary BSA necessary for SF medium in lymphocyte transformation by PHA, the basal SF medium was supplemented with four different concentrations ranging from 1.0 to 0.125% BSA and used for the lymphocyte transformation. As shown in Table I, addition of 1.0 to 0.25% BSA to the medium was found to be highly effective in inducing the PHA response of the lymphocytes ($P < 0.001$), whereas the addition of 0.125% BSA gave the response only at a level similar to that in BSA-free medium. On the other hand, in the absence of PHA, [^{3}H]thymidine incorporation in all the media containing different concentrations of BSA with the exception of 1.0% BSA was much lower than was the case with BSA-free medium. These results clearly show that extremely high stimulation indices could be obtained with the culture containing 0.25 and 0.5% BSA. Thus, for all subsequent experiments, SF medium supplemented with 0.25% BSA was used.

Time course of the lymphocyte transformation in SF medium. Lymphocyte transformations by different concentrations of PHA in SF medium and RPMI 1640 medium supplemented with 10% fetal calf serum during a period of 120 hr are summarized in Fig. 1. In RPMI 1640 medium, DNA synthesis began 24–48 hr after incubation and reached a maximum at 72 hr, whereas synthesis in SF medium began 48–72 hr and reached its maximum after 96 hr of incubation. The maximum value of DNA synthesis in SF medium was about four times higher than that in RPMI 1640 medium. The results also show that a high level of DNA synthesis was observed even with the lymphocytes in SF medium stimulated by PHA at a concentration as low as 0.6 μg/ml.

No effects of defatted BSA on lymphocyte transformation. It has been shown by one of the authors and his colleagues (7) that the BSA-bound fatty acids may play an important role in the growth of tumor cells in SF medium. Therefore, in order to determine whether the fatty acids have the same function on lymphocyte proliferation, we tested the PHA-stimulated transformation of lymphocytes in SF medium supplemented with

TABLE I. EFFECTS OF BSA CONCENTRATION ON DNA SYNTHESIS OF PHA-STIMULATED LYMPHOCYTES IN SERUM-FREE MEDIUM.[a]

Concentration of BSA (%)	[^{3}H]thymidine incorporation		Stimulation index
	PHA	Control	
1.0	359,224 ± 6,591[b]	3,092 ± 390	119.7
0.5	433,050 ± 17,871	746 ± 104	580.5
0.25	378,910 ± 11,741	383 ± 42	989.3
0.125	18,876 ± 2,876	389 ± 35	48.5
—	14,945 ± 2,690	1,989 ± 258	7.5

[a] In the basal SF media 5×10^5 lymphocytes supplemented with various concentrations of BSA (Armour) were incubated with 3.0 μg/ml of PHA or without PHA.

[b] Values represent the mean disintegrations per minute ± SD of incorporated [^{3}H]thymidine in triplicate cultures.

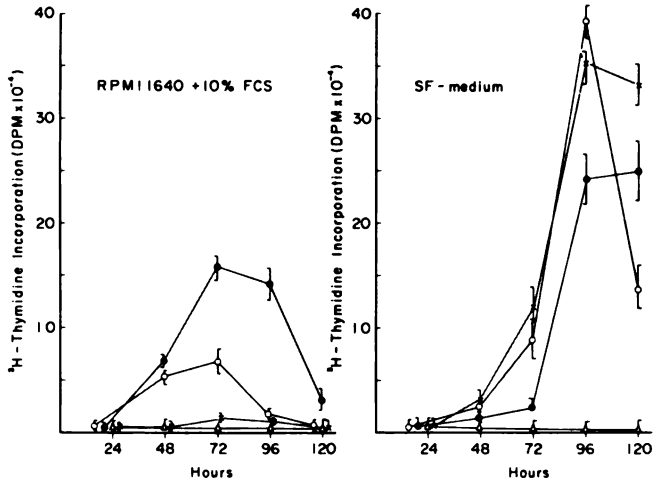

FIG. 1. Kinetics of PHA stimulation of human peripheral lymphocytes (5 × 10^5 cells) in SF medium and RPMI 1640 with 10% fetal calf serum. ●——●, 15.0 μg/ml of PHA; ○——○, 3.0 μg/ml of PHA; x——x, 0.6 μg/ml of PHA; Δ——Δ, no mitogen.

defatted BSA and compared the results with those in SF medium containing the native BSA. Different commercial preparations of native or defatted BSA (0.25%) were added to the basal SF medium and examined for their effect on the PHA response of the lymphocytes at a 96-hr incubation (Table II). In addition, transformation of lymphocytes by various concentrations of PHA in SF medium supplemented with 0.25% Armour's native or defatted BSA were measured at different incubation times (Fig. 2). Both the experiments clearly show that only one-tenth or less response to PHA of the lymphocytes was obtained in the SF medium supplemented with any preparations of defatted BSA. The decrease of medium pH was observed only with the culture in which a high level of incorporation of [^{3}H]thymidine was demonstrated.

We next examined the effect of oleic and linoleic acids on the PHA-stimulated transformation of lymphocytes. Both the fatty acids at concentrations of 1–4 μg/ml were added to the medium containing defatted BSA and tested for their effect on the PHA response of the lymphocytes. As shown in Fig. 3, addition of 1 and 2 μg/ml of fatty acids restored the transformation of lymphocytes to 60 and 75% of the control value, respectively, although high concentrations of the drugs (3 and 4 μg/ml) had no restoring effect on lymphocyte transformation and showed a somewhat inhibitory ef-

TABLE II. EFFECTS OF BSA AND DEFFATED BSA ON DNA SYNTHESIS OF PHA-STIMULATED LYMPHOCYTES.[a]

BSA	[^{3}H]thymidine incorporation		Stimulation index
	PHA	Control	
BSA (Fr.V)			
Armour	267,025 ± 32,043[b]	387 ± 56	690
Pentex	290,822 ± 18,868	375 ± 42	775
Sigma	328,345 ± 14,857	605 ± 37	543
Defatted BSA			
Armour	9,425 ± 1,074	161 ± 28	59
Pentex	11,094 ± 1,818	1,051 ± 156	11
Sigma	9,857 ± 1,003	409 ± 29	24

[a] In the basal SF media 5 × 10^5 of lymphocytes with 0.25% different commercial native or defatted BSAs were incubated with 3.0 μg/ml of PHA or without PHA.

[b] *Values represent the mean* disintegrations per minute ± SD of incorporated [^{3}H]thymidine in four cultures.

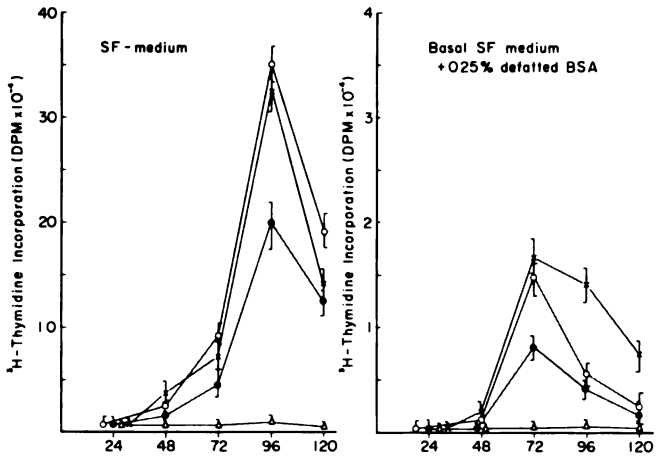

FIG. 2. Kinetics of PHA stimulation of human peripheral lymphocytes (5×10^5 cells) in the basal SF medium with 0.25% BSA and 0.25% defatted BSA. ●——●, 15.0 μg/ml of PHA; ○——○, 3.0 μg/ml of PHA; x——x, 0.6 μg/ml of PHA; △——△, no mitogen.

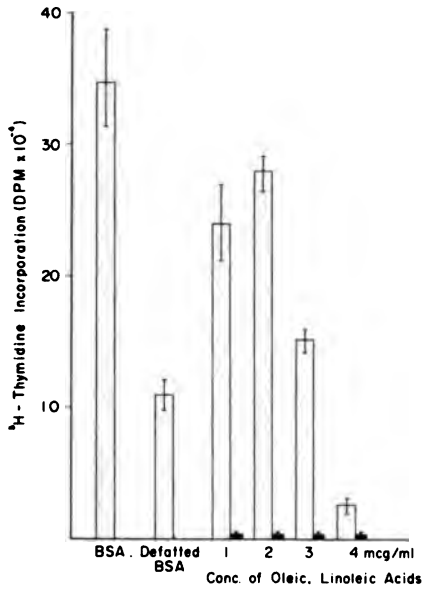

FIG. 3. The effects of oleic and linoleic acids on the DNA synthesis of PHA-stimulated lymphocytes. Lymphocytes, 5×10^5, were incubated with 3.0 μg/ml of PHA for 96 hr. □, [³H]thymidine incorporation in the medium supplemented with 0.25% BSA or defatted BSA with oleic and linoleic acids; ■, [³H]thymidine incorporation in the basal SF medium with oleic and linoleic acids.

fect. However, these fatty acids when added to the BSA-free basal medium showed no effect on the transformation (Fig. 3). These results clearly indicate that fatty acids may be indispensable to the medium component for the blast transformation of PHA-stimulated lymphocytes, although the collaboration of albumin with fatty acids may be needed for the transformation.

Discussion. SF medium formulated by Yamane *et al.* (7) has been shown to support the growth of tumor cells as well as or more abundantly than conventional media containing the serum. In the present experiments, it is clearly shown that this SF medium is also useful for the assay of DNA synthesis by PHA-stimulated lymphocytes. The results demonstrate the following characteristics of SF medium as a culture medium for lymphocyte transformation experiments: (a) remarkable response to lower PHA doses, (b) high incorporation of [³H]thymidine into the stimulated lymphocytes, and (c) low background resulting in a higher stimulation index.

One of the present authors has shown earlier that defatted albumin, unless reconstituted with its lipid extracts or oleic and linoleic acids, failed to support tumor cell growth in SF medium. Our experiments also clearly demonstrate that the albumin-bound fatty acids play an important role in transformation of the lymphocytes. By contrast, Polet and Spieker-Polet have reported that fatty acids may not be necessary for the transformation of lymphocytes stimulated by concanavalin A (Con A) (4, 5). The exact explanation for this discrepancy in the role of fatty acids in the transformation of lymphocytes is not clear. However, Polet

and Spieker-Polet performed the experiments in which Con A-treated lymphocytes were washed once with the medium containing 10% fetal calf serum, washed only once with the protein-free medium to remove free Con A, and then added to the fatty acid-free medium for the estimation of DNA synthesis. This procedure might allow binding to the cell surface of fatty acids derived from the fetal calf serum. Washing such cells only once also would not be sufficient to remove the cell-bound fatty acids. Therefore, it should be considered that the cells which retained fatty acids supported the transformation of lymphocytes, even if the fatty acids were not supplemented with the estimation medium.

The exact mechanisms of this augmentation by fatty acids of PHA-stimulated DNA synthesis of lymphocytes are not clear. However, it is well known that fatty acids in general play an important part in the physiological stability of cellular membranes of mammalian cells such as Ehrlich cells, polymorphonuclear leukocytes, and alveolar macrophages (9). Therefore, we presume that the fatty acids in this case also may play a part in the stabilization of lymphocyte and/or macrophage membranes.

Overall, it appears that the SF medium used in this work may be useful as a culture medium not only to test the mitogenic response of human lymphocytes for clinical studies but also to investigate the role of fatty acids in lymphocyte differentiation.

Summary. Employing a serum-free medium (SF medium), blast transforma human peripheral lymphocytes stim by PHA was investigated. A rema PHA response with extremely high st tion indices was obtained in SF mediu taining 0.25–0.5% BSA. However phocytes were unresponsive to PHA medium containing defatted BSA, supplied with oleic and linoleic acid concluded that the albumin-bounc acids play an important role in lymp transformation in SF medium, as ob in the case of tumor cell cultivation.

This work supported by a Cancer Resear from the Ministry of Education, Science and Japan.

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Received July 26, 1976. P.S.E.B.M. 1977, Vo

ı OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 449-452 (1977)

# of New Synthetic Analogs of Somatostatin on Gastric Secretion in the Chronic Fistula Dog (39691)

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ıtostatin (growth hormone release ng hormone), a peptide originally l from hypothalamic extracts, and responding synthetic tetradecapep- ıibit growth hormone release *in vitro* *vivo* [for review, see Vale *et al.* (1)]. ıstatin has been shown to inhibit gas- retion in patients with hypergastrine- l to reduce basal secretion and food- ɔgenous gastrin-stimulated secretion ɔcts that were free of gastrointestinal (2, 3). Subsequent investigations in d cats have demonstrated that soma- inhibits gastric secretion in response food and to exogenous gastrin (4-

structural requirements for the an- ory activity of somatostatin are not This report presents data relating to ibition of gastric secretion by soma- and a number of new synthetic ana- dogs with a gastric fistula wherein ɔn was evoked by pentagastrin.

ods and materials. Gastric secretion were conducted in animals from a of 50 female beagle dogs (8-10 kg) a chronic gastric fistula. Secretion ɔked by the intravenous infusion of a maximal secretory dose of pentagas- 5 μg/kg/hr) for 3 hr (−60-120 min). the experiment, the fistula was and outputs were collected continu- ɔamples were taken at 30-min inter- volume and acid concentration de- ıtions; the latter was obtained by ti- to pH 7 with 0.01 *N* NaOH. From lata, total acid output (milliequiva- ɣas calculated (volume × concentra- ɔata from placebo trials are reported nean ±SE.

wing a 1-hr period required to steady-state secretion during penta- stimulation (−60-0 min), somato- r its analogs were infused for 1 hr (0-60 min, 0.77 ml/min). Since there was little difference in their molecular weights, data are reported as the dose administered. The percentage reduction in volume, acid concentration and T.A.O. was determined from the means at each dose level by comparison to a placebo trial in the same animal. The percentage reduction in T.A.O. from each individual animal was used to determine relative potencies and the dose required to reduce output by 80% (ED_{80}, micrograms per kilogram per minute) according to the statistical procedures described by Finney (8).

Pentagastrin (gastrin pentapeptide, blocked) was obtained from Calbiochem, San Diego, Calif. Somatostatin and its analogs were synthetized at the Merck, Sharp and Dohme Laboratories, West Point, Pa. (Table I).

Results. During interdigestive periods, basal gastric acid output in dogs is minimal or absent. In 20 placebo experiments during steady-state secretion, pentagastrin infused at a rate of 2.5 μg/kg/hr produced a near maximal gastric secretory response. In a comparison of two placebo trials in the same animals, there was little variation in the concentration of acid secreted either within or between trials (129 ± 4 at 0 min to 125 ± 5 mequiv/liter at 120 min in trial A versus 133 ± 4 to 127 ± 5 mequiv/liter in trial B). The output volume decreased during the course of the experiment (24 ± 3 ml at 0 min to 16 ± 1 ml at 120 min versus 26 ± 3 to 18 ± 2 ml). T.A.O., the product of volume and concentration, followed the same time course and reflected the same variability as the volume measurements (3.08 ± 0.47 mequiv at 0 min to 2.06 ± 0.19 mequiv at 120 min versus 3.52 ± 0.44 to 2.42 ± 0.24 mequiv).

Somatostatin reduced gastric secretion evoked by pentagastrin. The dose calculated

1977 by the Society for Experimental Biology and Medicine
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ISSN 0037-9

TABLE I. PEPTIDE SEQUENCE OF SOMATOSTATIN AND ANALOGS.

Compound	Name	Molecular weight	Sequence
(A)	Somatostatin	1637.6	H—Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys—OH (3–14)
(B)	Des-(Ala¹,Gly²)-desamino-Cys³-somatostatin	1495.7	S—(CH₂CH₂CO—Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys—OH)
(C)	Des-(Ala¹,Gly²)-desamino-Cys³-dicarbasomatostatin	1458.37	CH₂—CH₂—CH₂—CH₂ (CH₂CO—Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser—NH—CH—COOH)
(D)	Des-(Ala¹,Gly²)-desamino-Cys³-decarboxy-dicarbasomatostatin	1413.6	CH₂—CH₂—CH₂—CH₂ (CH₂CO—Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser—NH—CH₂)
(E)	Des-(Ala¹,Gly²)-desamino-bis-(iNoc)-Lys⁴,⁹-dicarbasomatostatin	1728.8	CH₂—CH₂—CH₂—CH₂ (CH₂CO—Lys(iNoc)-Asn-Phe-Phe-Trp-Lys(iNoc)-Thr-Phe-Thr-Ser—NH—CH—COOH)
(F)	Bis-*S*-acetamidomethyl-dihydrosomatostatin	1781.7	H—Ala-Gly-Cys(Acm)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys(Acm)—OH

to reduce T.A.O. by 80% expressed as the ED_{80} (95% C.L.) was 0.06 μg/kg/min (0.02, 0.10). As shown by the data in Table II, somatostatin was about sixfold more effective in reducing the volume of gastric secretion (ED_{80} 0.07 μg/kg/min; 95% C.L., 0.04, 0.11) in comparison to the reduction in acid concentration (ED_{80} 0.41 μg/kg/min; 95% C.L. 0.23, 1.29). There was no significant difference in the potency in the reduction of volume (vol) and T.A.O. between somatostatin and the derivatives des-(Ala¹, Gly²)-desamino-somatostatin (B), ED_{80} vol = 0.03 (0.01, 0.31). T.A.O. = 0.08 (0.03, 0.23), des-(Ala¹, Gly²)-desamino-dicarbasomatostatin (C), ED_{80} vol = 0.06 (0.04, 0.14), T.A.O. = 0.04 (0.03, 0.07), and des-(Ala¹, Gly²)-desamino-decarboxy-dicarbasomatostatin (D), ED_{80} vol = 0.04 (0.03, 0.07), T.A.O. = 0.07 (0.02, 0.17). Although the derivatives cited above (compounds B, C, and D) were equieffective with somatostatin as inhibitors of output volume and T.A.O., they were less effective in reducing the acid concentration. Relative potencies to somatostatin for reduction in acid concentration could not be *determined* due to differences in the slope *of the dose-response lines.*

The peak inhibitory effect of somatostatin and the analogs occurred during the latter part of the infusion period (30 to 60 min of collection) and declined after termination of the treatment (30- to 60-min collection period). The duration of action of somatostatin and the analogs, as indicated by activity during the post-treatment period, was short. During the post-treatment period, it was not uncommon for a rebound effect to occur, and outputs exceeded values obtained during the corresponding placebo intervals (see footnote, Table II).

At 8 μg/kg/min, des-(Ala¹, Gly²)-desamino-bis-(iNoc)-Lys⁴,⁹-dicarbasomatostatin (E), an analog having both lysine amino groups protected and bis-*S*-acetamidomethyl-dihydrosomatostatin (F), wherein the cysteine³,¹⁴ groups were blocked by acetamidomethyl, were essentially ineffective as inhibitors of gastric secretion. No side effects were noted with somatostatin or the analogs.

Discussion. Somatostatin is a potent inhibitor of gastric secretion in both man (2, 3) and experimental animals (4–7). However, the published data indicate only changes in T.A.O. and do not specify if the effect was on output volume or acid concen-

TABLE II. PERCENTAGE CHANGE IN PENTAGASTRIN-EVOKED GASTRIC SECRETION IN CHRONIC FISTULA DOGS[a] (MEAN ± SE)

Compound	Dose[b] (μg/kg/min)	No. dogs	Volume		Acid concentration		Total acid output	
			Peak[c]	Post[d]	Peak[c]	Post[d]	Peak[c]	Post[d]
Placebo	0.0	20	+12 ± 17	± 13 ± 10	+1 ± 4	+5 ± 3	+23 ± 19	+15 ± 12
Somatostatin	0.80	8	−95 ± 2	−76 ± 10	−78 ± 11	−56 ± 11	−99 ± 1	−85 ± 6
(A)	0.20	8	−94 ± 2	−44 ± 18*	−72 ± 12	−33 ± 11*	−97 ± 2	−50 ± 19
	0.08	6	−90 ± 4	−17 ± 31*	−52 ± 6	−28 ± 10*	−94 ± 2	−28 ± 40*
	0.04	7	−81 ± 7	−12 ± 24*	−31 ± 11	−1 ± 12*	−83 ± 8	−2 ± 45*
	0.02	4	−23 ± 17*	+30 ± 31**	−29 ± 13*	−15 ± 7*	−33 ± 21*	+2 ± 21*
	ED_{50}(μg/kg/min)		0.07 (0.04, 0.11)		0.41 (0.23, 1.29)		0.06 (0.02, 0.10)	
(B)	2.40	4	−97 ± 2	−94 ± 4	−54 ± 20	−54 ± 22	−97 ± 2	−98 ± 1
	0.80	4	−96 ± 3	−62 ± 21	−52 ± 19	−40 ± 18*	−97 ± 2	−67 ± 24
	0.20	4	−97 ± 2	−32 ± 20*	−42 ± 20	−26 ± 7	−97 ± 2	−47 ± 17
	0.05	4	−95 ± 2	−35 ± 18*	−47 ± 12	−15 ± 6	−96 ± 2	−43 ± 18*
	0.0125	6	−68 ± 9	−28 ± 18*	−19 ± 21*	−14 ± 6	−68 ± 10	−43 ± 18
	0.006	6	−13 ± 35*	−4 ± 8*	−14 ± 14	−3 ± 4*	−18 ± 36*	−6 ± 9
	ED_{50}(μg/kg/min)		0.03 (0.01, 0.31)				0.08 (0.03, 0.23)	
(C)	0.80	4	−94 ± 1	+1 ± 28*	−63 ± 21	−32 ± 8	−93 ± 3	−36 ± 14
	0.20	4	−90 ± 3	+14 ± 27*	−51 ± 9	−3 ± 9	−96 ± 3	+12 ± 26*
	0.04	4	−63 ± 16	+32 ± 29*	−38 ± 21*	−7 ± 7*	−67 ± 17	+17 ± 18*
	0.01	4	−3 ± 29*	+8 ± 35*	−5 ± 18	−1 ± 10	−2 ± 37*	+15 ± 44*
	ED_{50}(μg/kg/min)		0.06 (0.04, 0.14)				0.04 (0.03, 0.07)	
(D)	0.80	4	−86 ± 10	−60 ± 10	−49 ± 19*	−38 ± 16	−97 ± 2	−62 ± 13
	0.20	4	−91 ± 5	−24 ± 35*	−67 ± 12	−17 ± 5	−96 ± 3	−32 ± 34*
	0.05	4	−83 ± 4	−31 ± 10	−13 ± 10*	−5 ± 7	−88 ± 5	−42 ± 6
	0.025	4	−59 ± 14	−16 ± 29	−14 ± 16*	−24 ± 19*	−59 ± 15	−40 ± 19*
	0.0125	4	+28 ± 91*	+29 ± 15**	−18 ± 5	−6 ± 6	+11 ± 76*	+22 ± 19
	ED_{50}(μg/kg/min)		0.04 (0.03, 0.07)				0.07 (0.12, 0.17)	

[a] Pentagastrin, 2.5 μg/kg/hr (−60 to 120 min).
[b] Dose infused, 0 to 60 min.
[c] Peak effect during treatment (30- to 60-min collection period).
[d] Effect during 30-min post-treatment period (60–90 min).
* Includes values greater than during placebo trial.
** All values greater than during placebo trial.

tration. Reduction in acid concentration would serve to elevate intragastric pH and have potential use in ulcer therapy while reduction of volume alone may not achieve this effect. In the present experiments in the dog, volume was more readily reduced by somatostatin and a sixfold higher dose was required to lower acid concentration.

The inhibitory effects of somatostatin were of short duration and this, along with other biological activities, namely, inhibition of the release of growth hormone, insulin and glucagon (9), may limit its clinical utility in acute hypersecretory states and as an anti-ulcer agent. There is one clinical report of the successful control by somatostatin of a patient with intractable gastrointestinal bleeding. However, during treatment, there was a marked elevation in plasma glucose which responded to administered insulin (10).

In a separate unpublished study in animals from the colony used in the experiments reported herein, somatostatin did not significantly alter plasma glucose, and plasma insulin (radioimmunoassay) decreased from 9 to 3 μU/ml in two of four dogs given somatostatin at 0.8 μg/kg/min for 1 hr. It would therefore appear that adjustment of the dose could allow suppression of gastric secretion without substantially altering blood glucose.

In order to prepare an analog of somatostatin with selectivity for gastric secretion, it was appropriate first to determine some of the structural requirements for antisecretory activity. As with somatostatin, the active analogs were somewhat more potent in reducing volume output than acid concentration. The two amino terminal residues, alanine and glycine, and the remaining terminal group of cysteine were not essential for inhibition of gastric secretion as determined by the effect of des-(Ala[1], Gly[2])-desamino-

somatostatin (B) on T.A.O. It has also been reported that these changes do not alter inhibition of growth hormone release (11). Activity was lost when ring formation was prevented by protection of the thiol groups of the cysteine residues of dihydrosomatostatin (F). This type of modification also caused loss of inhibition of growth and glucagon and insulin release *in vivo* (11).

Analogs locked in a cyclic structure as in des-(Ala^1, Gly^2)-desamino-dicarbasomatostatin (C) and des-(Ala^1 Gly^2)-desamino-decarboxy-dicarbasomatostatin (D) were active. Activity in compound D also indicated that the terminal carboxyl group was not required. However, the importance of the ϵ-amino groups was demonstrated by the loss of activity when they were blocked by isonicotinyloxycarbonyl (compound E).

As with somatostatin, the active analogs (B, C, and D) had a short duration of action. The active analogs differed in that the slope of their dose–response curve for reduction of acid concentration was less steep than that obtained for somatostatin. Since they were less effective in reducing acid concentration, the analogs would also be expected to be less effective in elevating intragastric pH.

Summary. Somatostatin is a potent inhibitor of pentagastrin-evoked gastric secretion in the dog. Exploration of some of the structural requirements for antisecretory activity in this species has revealed that potency in reducing T.A.O. has been retained in compounds wherein alanine (Ala^1) and glycine (Gly^2) and the amino and carboxyl groups of terminal cysteine^{3,14}, have been omitted. Locking the ring structure by replacing the sulfur with carbon retained activity. Compared to somatostatin, the active analogs (des-(Ala^1, Gly^2)-desamino-Cys^3-somatostatin, des-(Ala^1, Gly^2)-desamino-Cys^3-dicarbasomatostatin, and des-(Ala^1, Gly^2)-desamino-Cys^3-decarboxy-dicarbasomatostatin)

: OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 453-456 (1977)

d H_2-Histamine Receptor Antagonists and Protection against Traumatic Shock[1] (39692)

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since the classic work of Sir Henry nd Sir Thomas Lewis, histamine has nplicated by many workers in lethal- ı circulatory shock and trauma (1-6). ecently, it has been proposed that a formed "intrinsic histamine" synthe- y endothelial and/or vascular smooth cells may contribute to lethality in yndromes (7, 8). In contrast to these here is evidence which indicates that tment of animals with histamine ɔ shock may actually protect animals than exacerbate mortality (9, 10). ough H_1-receptor antihistamines een utilized from time to time to the possible contribution of hista- ı different forms of circulatory shock 1), there is not to our knowledge any hensive study available which has l several different forms of these an- nines over a wide dose range in a led shock model, nor have any stud- n done with H_2-receptor antagonists le-body traumatic shock. It would be ıry to examine both kinds of hista- ntagonists since information is accu- ıg which suggests that both kinds of ne receptors may be important in ion of the cardiovascular system (12, urthermore, it is distinctly possible stamine exerts differential receptor in shock. The studies herein explore of both H_1- and H_2-receptor block- l suggest that histamine may be in- in more than one way in traumatic

ods. Adult male mice of the ICR strain (Royalhart Laboratory Animals, Inc., New Hampton, N.Y.), weighing 26-28 g and lightly anesthesized with pentobarbital sodium (Nembutal, 2 mg/100 g, ip), were subjected to 350 revolutions of Noble-Collip drum trauma (NCDT) at 40 rpm (14). NCDT was chosen since it has been shown to be readily amenable to quantitation of the imposed stress in mice (14). In other terms, by varying the number of revolutions of the drum, a specific degree of hypotension and survival can be produced. ICR mice were specifically utilized because they are known to be sensitive to the toxic effects of histamine (15). Different groups of mice were pretreated ip 50 min prior to NCDT with various doses (i.e., 1, 10, and 25 mg/kg) of an antihistamine. Each antihistamine was dissolved in normal isotonic saline solution. The volume injected in all cases was 1 ml/100 g. Unpretreated controls were always subjected to NCDT simultaneously with the experimentals. Two groups of untraumatized mice were pretreated with high doses of antihistamines (e.g., 25 or 100 mg/kg). The H_1-receptor blockers utilized in this study were: diphenhydramine hydrochloride (Parke Davis and Co.), chlorpheniramine maleate (Schering Corp.), promethazine hydrochloride (Wyeth Laboratories), pyrilamine maleate (K & K Laboratories), and pyribenzamine hydrochloride (Ciba Pharmaceuticals). The H_2-receptor antagonist, burimamide hydrochloride, was a gift from Dr. J. W. Black (Smith, Kline and French Laboratories). Cumulative mortality was monitored for 120 hr. All animals were grossly autopsied at death for characteristic signs of shock (14, 16, 17). Mice showing fractured skulls, subdural hematoma, lacerated viscera, or torn vessels in the thoracic or abdominal cavities were not included; less than 3% of the animals exhibited such injuries. The data were analyzed for statisti-

work was supported by U.S. Public Health grants, No. HL-18015 and HL-18002.

uests for reprints should be sent to B. M. Department of Physiology, Box 31, SUNY, te Medical Center, Brooklyn, New York

977 by the Society for Experimental Biology and Medicine
ved.

ISSN 0037-97

TABLE I. INFLUENCE OF ANTIHISTAMINES ON DRUM TRAUMA MORTALITY.

Therapy	Dose (mg/kg)	N	Cumulative mortality (%) 2 hr	72 hr	120 hr
Controls	–	34	38	38	38
Diphenhydramine	1	20	5[a]	5[a]	5[a]
	10	20	5[a]	15	15
	25	20	15	15	15
Promethazine	1	23	4[a]	13[c]	13[c]
	10	20	10[c]	10[c]	10[c]
	25	25	4[a]	4[a]	4[a]
Pyrilamine	1	19	11[c]	32	32
	10	19	11[c]	26	37
	25	19	11[c]	11[c]	11[c]
Pyribenzamine	1	20	0[a]	10[c]	25
	10	19	11[c]	42	53
	25	19	11[c]	32	37
Chlorpheniramine	1	20	30	65	90[d]
	10	18	44	89[d]	89[d]
	25	18	78[a]	83[a]	94[d]
	(25)[b]	28	0[d]	0[d]	0[d]
Burimamide	1	18	44	61	67
	10	20	30	45	60
	100	18	94[d]	94[d]	94[d]
	(100)[b]	18	0[a]	0[a]	0[a]

[a] Significantly different from controls ($P < 0.01$).
[b] Animals in this group were not subjected to trauma.
[c] Significantly different from controls ($P < 0.05$).
[d] Significantly different from controls ($P < 0.001$).

cal significance by use of a one-way χ^2 test. A total of 550 mice was utilized for these studies.

Results. A scan of the data in Table I reveals that different antihistamines exerted a differential effect on mortality resulting from NCDT in mice.[3] Four of the five H_1-receptor antagonists exerted significant protection against death resulting from the trauma. All of the H_1-receptor antagonists except chlorpheniramine resulted in significant protection early after trauma, i.e., at 2 hr. Promethazine was the only H_1-receptor antagonist that resulted in significant protection beyond 72 hr at all dose levels. It should be noted that there appears to be a reverse dose-response relationship with diphenhydramine in contrast to the other antihistamines used.

[3] We are indebted to Dr. Patrick L. Ross, Department of Psychiatry and Psychology, Nassau County Medical Center (East Meadow, New York), for aiding us in the statistical calculations presented in this manuscript.

Pretreatment with the H_2-receptor antagonist, burimamide, as well as chlorpheniramine (an H_1-receptor blocker) was associated, at many dose levels, with an exacerbation of mortality. Mortality was, however, not seen in the two groups of untraumatized animals which were pretreated with high doses of chlorpheniramine (25 mg/kg) and burimamide (100 mg/kg).

Discussion. To our knowledge, this is the first report which demonstrates that at least four different H_1-receptor antagonists exert significant protection in a form of circulatory trauma other than anaphylaxis or endotoxemia. Since the early 1930s, plasma histamine levels have been shown to increase early after several different forms of circulatory stress, e.g., burns (1, 2, 6), anaphylaxis (2), endotoxemia (4, 5), hemorrhage (18), and NCDT (19). Tissue levels of histamine decarboxylase are also known to be increased in several forms of circulatory stress and trauma (7, 8, 19).

The response of the cardiovascular system to histamine depends upon the existence,

rtion, of at least two different or histamine, H_1- and H_2-recep- 3). However, in most species so ated, the dilator responses of mi- ›ry blood vessels as well as the nd chronotropic responses to his- thought to be mediated primarily ptors (12, 13). H_1-receptor an- re known to be capable of com- :king the contractile responses of ›n large arterial and venous ves- 1) and to block partially the de- ions of histamine on microcircu- d vessels (2, 12, 13, 20, 22). A echanism for the protective ac- $_1$-receptor antagonists in NCDT :refore, be attributed to the of excessive vasoconstriction out by either released or newly tamine. It is thought that exces- nstriction may be a major cause in many shock syndromes (23). lso entertain the possibilities that r antagonists could: (i) indirectly istamine mediated release of cat- s from the adrenal medulla (2), eventing excessive vasoconstric- ›r (ii) prevent the histamine-in- eases in capillary permeability is known to be associated with a ›f plasma from the intestinal cap- into the interstitial fluid spaces :oncentration (14, 16, 24). Ro- ; from NCDT also exhibit micro- failure (24).

ata in this report indicate that ion of either chlorpheniramine ıide exacerbate mortality follow- . It is of interest to note that ·amine, of all antihistamines so ated in the mouse skin microcir- roduces potent vasoconstriction ›vessels (i.e., precapillary sphinc- terioles, arterioles, and venules) ldition, chlorpheniramine is the istamine of those used here ıduce venular stasis and rhexis of ›illary venular walls in the mouse ation (25). These specific phar- actions of chlorpheniramine ıportant contributing factors ac- ›r the increased mortality seen a in our experiments. It is note- worthy that chlorpheniramine can antagonize the coronary dilatating action of histamine (26), as do H_2-receptor antagonists (27). Such data could be used to suggest that chlorpheniramine may have H_2-receptor blocking properties.

The action of released (or newly formed histamine) on H_2-receptors in NCDT may be important for survival since the specific H_2-receptor blocker, burimamide, enhances mortality at the highest dose level studied. Histamine induced vasodilatation via H_2-receptors may thus be a beneficial effect in NCDT. In this context, it is interesting to note that histidine decarboxylase activity is activated in NCDT, presumably at the microcirculatory level rather than in large blood vessels (19). Although our data could therefore indirectly be used as support for the contention that activation of histidine decarboxylase may be beneficial in certain shock syndromes (7, 8, 19), this will have to await further investigation. Other factors that will have to be contended with in future investigations are the inverse dose-response relationships observed with diphenhydramine, the possibility of H_2-receptor antagonism by H_1-receptor blockers at higher doses, and the possibility of local anesthesia induced by high doses of antihistamines (20).

Summary. Pretreatment of mice with four different H_1-receptor antagonists (i.e., diphenhydramine, promethazine, pyrilamine, and pyribenzamine) exerted significant protection against Noble-Collip drum trauma (NCDT). Blockage of H_2-receptors, however, was associated with an exacerbation of mortality after NCDT. These results suggest that certain actions of histamine on H_2-receptors could be beneficial in NCDT in mice, while actions on H_1-receptors may be detrimental.

The authors are indebted to Mr. C. Monnell and Mr. A. Ogunkoya for their excellent technical assistance.

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Received October 16, 1975. P.S.E.B.M. 1977, Vol 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 457-460 (1977)

ce of Meal Frequency and Timing on Physical Activity and Body Weights of Rats[1] (39693)

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ing human beings to eat a 2000-kcal the morning resulted in a greater oss than when the 2000-kcal meal sumed in the evening (1). These als were physically active during the inactive at night. In other studies it ed that humans exercised after a onsume more oxygen than can be ed for by the additive effects of the d the exercise (2-4). Mice allowed nly at the onset of darkness each , at the onset of their usual active ained less weight than mice forced t the onset of light (5). Also rats o eat from 8 to 10 AM, which is heir usual period of inactivity, are ficient than *ad libitum* fed rats in ng food energy to body weight gain hese observations suggest that the f the meal relative to periods of activity may be an important deter- n the regulation of energy metabo-

urpose of the experiments reported s to examine the influence of meal- elative to the pattern of physical on body weight gain and epididymal weights of rats. Rats were fed three diets either at the beginning of ial period of inactivity (8 AM) or at nning of their usual period of high activity (7 PM). Meters were uti- nonitor physical activity of the rats. *als and methods*. For study, male -Dawley rats[2] were housed in indi- etal cages (18 × 24 cm) with raised rs or in plastic shoe-box cages (20) with wood shavings as bedding. l rats were allowed to eat from 8 AM to 10 AM or from 7 PM to 9 PM. Water was available *ad libitum*. Room lights were on from 7 AM to 7 PM. Body weights of the rats were recorded just before the meal or for *ad libitum* fed rats at 10 AM. Three semipurified diets were fed (Table I). Five experiments were conducted.

Activity meters[3] were utilized to obtain estimates of physical activity. Rats housed in shoe-box cages were placed on the top plate of a meter. Animal movements were sensed as the animal moved about the cage, and their movements were recorded on a digital readout. Four meters were utilized. An animal was on one meter for 24 hr and then rotated to another meter. Movements of all animals were recorded on each meter during an experiment; thus any sensitivity differences among the four meters in detecting movements would not influence the treatment results. A constant and minimal quantity of bedding was added to each cage when activity was recorded.

In experiments III-V, rats were killed to obtain an estimate of epididymal fat pad weights. Animals fed from 8 to 10 AM were killed at 8 AM, and animals fed from 7 to 9 PM were killed at 7 PM.

Rats were pair-fed in experiments III-V. Based on the food intake of animals fed from 8 to 10 AM, animals were given an equal amount of food at 7 PM. These rats generally consumed their food within 30 min.

Results. Rats, weighing 250 g initially, were fed from 8 to 10 AM or were allowed *ad libitum* access to Diet 1 for 4 wk. Their physical activity was then recorded (Table II). Based on estimates obtained with the activity meters, the total daily movements of the rats were not influenced by the meal pattern. Regardless of meal pattern, the rats were most active during the dark hours.

orted by Grant No. NIH 15847 and by Reeer Development Award No. NIH K04 AM DRR. Michigan Agricultural Experiment ournal Article No. 7705.

[2] an Research Animals Inc., Haslett, Michigan.

[3] Columbus Instruments Co., Columbus, Ohio.

1977 by the Society for Experimental Biology and Medicine rved.

ISSN 0037-9727

Forcing the rats to eat during the light hours did alter their circadian pattern of physical activity; however, 68% of their activity still occurred during the dark hours.

In a second experiment, rats were fed a 2-hr meal during the light hours or during the dark hours. Total physical activity was not influenced by meal pattern (Table III). Rats fed in the morning were more active from 8 to 10 AM than rats fed in the evening. Again, 70–75% of the rats' activity occurred during the dark hours.

Food intake and body weights of rats offered a high-carbohydrate diet from 8 to 10 AM or from 7 to 9 PM (experiments II and III) were recorded. Food intake and body weight gain of these rats was not influenced by the feeding pattern (Table IV). In experiment III, one group of rats was also pair-fed. Body weight gain of these rats was similar to that observed in rats fed from 8 to 10 AM or from 7 to 9 PM. Likewise, the epididymal fat pad weights of these rats were not influenced by the feeding pattern.

It is possible that the composition of the diets fed to the rats might influence the relationship of meal-timing and physical activity to body weight gain. In experiments IV and V, diets containing intermediate and high levels of fat were fed (Table IV). Again, neither body weight gain nor epididymal fat pad weights were influenced by the timing of the meal.

TABLE I. COMPOSITION OF THE DIETS.

	Diet		
	I (g)	II (g)	III (g)
Basal[a]	33.9	33.9	33.9
Glucose	66.1	33.0	0.0
Tallow	0.0	15.0	30.0
Total	100.0	81.9	63.9

[a] The basal mix contained (in grams/33.9 g): casein, 20.0; methionine, 0.3; mineral mix, 4.0 [see Ref. (7)]; vitamin mix, 0.4 [see Ref. (7)]; choline chloride, 0.2; cellulose, 4.0; and corn oil, 5.0.

Discussion. Rats allowed to eat from 8 to 10 AM appear to be more efficient than *ad libitum* fed rats in converting diet consumed to body weight gain (7, 8). In a previous report, spontaneous activity of the rats was determined in revolving cages (7). Meal-fed rats exhibited a reduced spontaneous activity level. It was suggested that the reduced activity of the meal-fed rats might account for their improved energy efficiency. However, in the present study, total activity of the meal-fed rats as estimated with electronic activity meters was not reduced. These results would suggest that factors other than alterations in physical activity are involved in the improved food efficiency of the meal-fed rat. Unfortunately, data obtained with the activity meters did not allow calculation of actual energy expenditure associated with physical movement but, rather, gave only an estimate of the relative activity among the two groups of rats. It has been noted that physical activity contributes relatively little to the total daily energy expenditure of the rat (3, 6, 10).

A shift in the meal from 8–10 AM to 7–9 PM influenced the pattern of physical activity of the rats only minimally (Table III). However, in neither animals allowed to eat a high-carbohydrate diet in the morning or in the evening nor in pair-fed animals did the timing of the meal influence body weight gain or epididymal fat pad weights.

Consumption of the intermediate and high-fat diets should have altered the

TABLE II. PHYSICAL ACTIVITY OF MEAL-FED AND *ad libitum* FED RATS, EXPERIMENT 1, DIET I[a].

	Total activity per day	Percentage of total		
		8–10 AM	10 AM–7 PM	7 PM–7 AM
Fed 8–10 am	19,203 ± 515	13.2 ± 1.4	18.0 ± 1.5	67.7 ± 2.3
Ad libitum fed	18,358 ± 584	2.4 ± 0.9[b]	9.2 ± 2.3[b]	87.4 ± 3.6[b]

[a] Mean ± SEM of activity meter recording per day for eight rats. Activity of each rat was determined on 4 separate days from Day 28 to 44 of the experiment. Meal-fed rats weighed 365 ± 11 g after 28 days, gained 36 ± 3 g, and consumed 129 ± 10 g food from 28 to 35 days whereas *ad libitum* fed rats weighed 411 ± 9 g after 28 days, gained 33 ± 4 g, and consumed 154 ± 8 g food from 28 to 35 days on the experiment.

[b] *Meal fed versus ad libitum* fed, significantly different, $P < 0.05$.

TABLE III. PHYSICAL ACTIVITY OF RATS MEAL-FED IN THE MORNING OR IN THE EVENING, EXPERIMENT II, DIET I[a].

Time of feeding	Total activity per day	Percentage of Total			
		8–10 AM	10 AM–7 PM	7 PM–9 PM	7 PM–7 AM
8–10 AM	15,042 ± 532	10.7 ± 0.7	17.9 ± 0.9	16.2 ± 1.1	69.7 ± 3.2
7–9 PM	14,135 ± 863	4.3 ± 0.5[b]	19.1 ± 1.1	16.4 ± 1.4	75.3 ± 4.4

[a] Mean ± SEM of activity meter recordings per day for eight rats. Activity of each rat was determined on 4 separate days from Day 19 to 35 of the experiment. Body weight and food intake data are presented in Table IV.
[b] AM versus PM, significantly different, $P < 0.05$.

TABLE IV. EFFECT OF MEAL-TIMING ON FOOD INTAKE, BODY WEIGHT GAIN, AND EPIDIDYMAL FAT PAD WEIGHTS IN RATS[a].

	Time of Meal		
	8–10 AM	7–9 PM	7 PM – pair-fed to AM rats
Experiment II (Diet I)			
Initial weight (g)	180 ± 3	180 ± 3	–
Food intake (g)	473 ± 22	486 ± 15	–
Body weight gain (g)	93 ± 11	88 ± 7	–
Experiment III (Diet I)			
Initial weight (g)	220 ± 4	219 ± 5	220 ± 5
Food intake (g)	520 ± 20	560 ± 32	520 ± 20
Weight gain (g)	92 ± 6	114 ± 7	93 ± 8
Epididymal fat pad (g/100 g body wt)	122 ± 0.07	1.17 ± 0.08	1.29 ± 0.07
Experiment IV (Diet II)			
Initial weight (g)	236 ± 3	–	235 ± 3
Food intake (g)	547 ± 30	–	544 ± 28
Weight gain (g)	112 ± 7	–	123 ± 5
Epididymal fat pad (g/100 g body wt)	1.71 ± 0.08	–	1.84 ± 0.07
Experiment V (Diet III)			
Initial weight (g)	235 ± 4	–	236 ± 3
Food intake (g)	480 ± 25	–	478 ± 27
Weight gain (g)	148 ± 8	–	146 ± 12
Epididymal fat pad (g/100 g body wt)	2.38 ± 0.14	–	2.59 ± 0.21

[a] Mean ± SEM for 10 rats. Rats in experiment II were fed for 5 weeks whereas rats in experiments III–V were fed for 6 weeks.

plasma insulin to glucagon ratio in the rats. However, the body weight gains and epididymal fat pad weights of rats fed these diets were not influenced by the timing of the meal. Thus, the reported calorigenic action of glucagon (9) did not appear to be potentiated by a shift in the rats' meal pattern.

The reported synergistic action of meal-timing and physical activity on energy metabolism in humans (1–4) was not evident in the present study. The methods utilized (body weight gain and epididymal fat pad weights) may have lacked the sensitivity necessary to detect possible effects; however, body weight changes were evident in humans within one week after shifting the meal pattern. Alternatively, the rat may not be an appropriate animal model for such studies since the energy cost of physical activity for the rat is a negligible proportion of its total energy requirement (3, 6, 10). Carefully controlled long-term studies in humans or in animals with body weights similar to humans are needed to evaluate the possible interaction of meal-timing and physical activity in the control of energy balance.

Summary. Rats fed from 8 to 10 AM exhibited the same total physical activity per day as did *ad libitum* fed rats. Likewise rats fed from 7 to 9 PM did not alter their total activity. As expected, all rats were most

active when the lights were out. A shift in time of feeding did not influence body weight gain or epididymal fat pad weights of the rats. An interaction between meal-timing and physical activity in the control of body weight was not evident in the present study with rats.

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Received May 28, 1976. P.S.E.B.M. 1977, Vo

NGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 461-466 (1977)

:t of Maturation on Capillary Density, Fiber Size and Composition in Rat Skeletal Muscle[1] (39694)

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most mammals, postnatal growth of tal muscle appears to be due to a hy- ɔphy of existing fibers rather than to ·plasia (1). This means that the fibers n length and in girth, but their number not increase with maturation. Because :letal muscle the capillaries run parallel ıd the fibers these vessels are pushed by the fibers as they grow. If the num- f capillaries per muscle fiber remains ant, the increase in body weight should : in a decrease in capillary density, i.e., umber of capillaries per mm². Differ- in capillary density associated with weight have been recognized by some tigators (2, 3), but overlooked by oth- hen interpreting their experimental re- (4).

: have studied capillary density and le fiber size and composition in three les of the rat in order to quantitate the ɣes that occur with maturation.

terials and Methods. Forty-two female ɣue-Dawley derived rats[3] weighing be- ı 99 and 291 g were studied in Denver, altitude of 1610 m above sea level. ınimals were kept in plastic cages at a erature of 23 ± 1° and given water and (Purina chow) *ad lib.* The rats were ıed just before the experiments.

ree muscles were selected for study: ɔleus, the tibialis anterior and the gas- emius (medial head). These muscles dissected out after the animals had anesthetized with sodium pentobarbi- ɨ0 mg/kg) administered intraperitone- The rats were sacrificed with a pento- tal overdose. Immediately after dissec- he muscles were weighed on a Mettler electronic scale (Model P1200). The gastrocnemius and the plantaris were weighed together. The medial head was then separated. The muscles were cut transversely at the widest point of the belly, where the cross section is usually the largest, and frozen in isopentane cooled with liquid N_2 at a temperature of −130°, while attached to a cork using tragacanth gum. We found no difference in weight in samples weighed before and after freezing. Samples were stored in a freezer at a temperature of −70° before slicing at a thickness of 15-20 μm in a cryostat (Type HR, Slee International, Inc., Pennsauken, N.J.) at −20°.

The calcium method of Padykula and Herman (5) demonstrates the adenosinetriphosphatase (ATPase) activity in tissue sections. Tissues with active ATPase systems are incubated in a medium containing ATP and a calcium salt. The calcium phosphate that forms is then made visible by cobalt chloride and ammonium sulfide producing insoluble dark cobalt sulfide which color intensity is proportional to the enzyme activity. The ATPase systems in skeletal muscle exhibit different pH lability which permits the identification of fiber types (6). We have used the ATPase method with preincubation at a pH of 4.4 and 9.4. In the rat the red fibers are those with the highest oxidative capacity and highest oxidative enzyme activity, the white fibers have the lowest oxidative capacity and the intermediate fibers are those with medium oxidative capacity (7). In all three muscles the strongest ATPase activity, after preincubation at a pH of 4.4, indicated by an intense black color, was seen in the intermediate fibers, while the lowest ATPase activity, indicated by a light sepia was seen in the red fibers. White fibers exhibited intermediate ATPase activity in the tibialis anterior and the gastrocnemius. A fourth type of fiber, possessing high oxidative capacity, shown

pported by USPHS, National Institute of ı, Grant Nos. HL 12679, HL 18145 and GRS

ıpported by a fellowship from the Rockefeller ation.

nonsen Labs., Inc., Gilroy, California.

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ISSN 0037-

by high DPNH tetrazolium reductase activity, but with intermediate ATPase activity after preincubation at a pH of 4.4 can occasionally be recognized in the soleus (6). We have included this fourth fiber type with the red fibers when reporting composition in this muscle.

In the gastrocnemius and tibialis anterior, photomicrographs were taken, using a Leitz Orthomat system, of areas where three fiber types were readily discernible. In the soleus, two photographic fields usually covered the whole cross section of the muscle. These photomicrographs were then projected, at a known magnification, on a screen to estimate, by stereology using the differential point-counting method, the area occupied by the different types of fibers (8). The absolute number of fibers of each type per unit area was also counted. With this information the average muscle fiber cross sectional area was calculated. The average number of fibers counted per animal was 1071 in the soleus, 2325 in the gastrocnemius and 2921 in the tibialis anterior. With these large samples the use of sterology was justified.

The Gomori technic for alkaline phosphatase (9) using β-glycerophosphate in barbital buffer at a pH of 9.2 and the ATPase reaction, after preincubation at a pH of 3.8 were used to identify capillaries. These two technics permit the visualization of capillaries by reacting with the capillary wall. No differences in capillary counts were found when these two methods were employed on serial cross section slices of the same muscle from the same animal. In each muscle, 30 different microscopic fields, selected at random over the areas where fiber composition was studied, were used to count capillaries under oil immersion (1000X). Each field had an area of 0.0268 mm^2 (Fig. 1). These direct counts were then averaged and the values for capillary density were expressed as capillaries per mm^2. Capillary to fiber ratios for each muscle were calculated from the values for capillary and fiber densities.

Results. The weight of the three muscles increased linearly with body weight as seen in Fig. 2. The correlation between these variables was strong. On the average, the weight of the gastrocnemius, tibialis anterior and soleus increased by 272, 229, and 206%, respectively with increases in body weight from 100 to 300 g. Figure 3 shows that the average cross sectional area of the fibers, at a given body weight, was different in the three muscles. It was the largest in the soleus, the smallest in the tibialis anterior, and intermediate in the gastrocnemius. In each muscle the cross sectional area of the

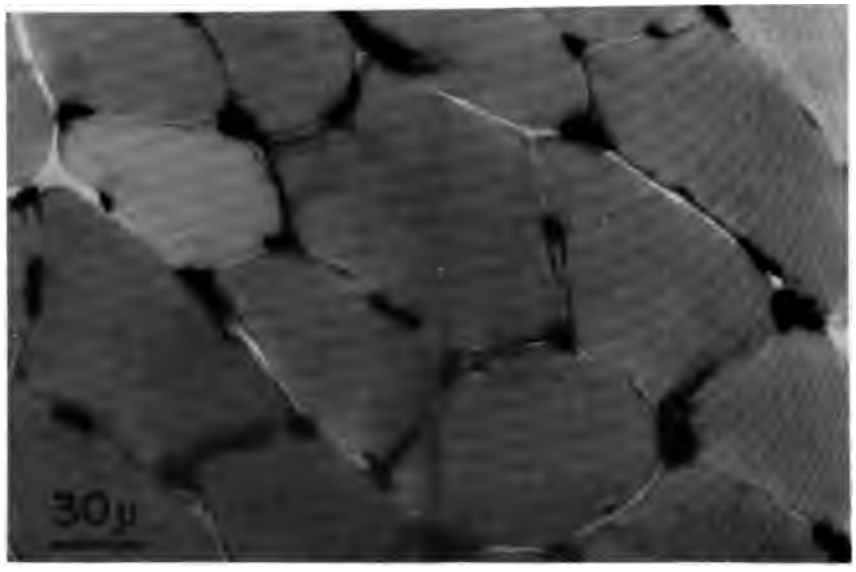

FIG. 1. Photomicrograph of cross section of rat soleus processed for ATPase after preincubation at a pH of *3.8. Capillaries are stained* in black.

ibers was linearly related to the body veight of the rats. The correlation between hese two variables was also strong. The ncrease in cross sectional area of the fibers ›ver the range from 100 to 300 g body veight was less in the tibialis anterior than n the other two muscles.

As illustrated in Fig. 4, at a given body veight, the capillary density was signifi- antly higher in the tibialis anterior than in he gastrocnemius and the soleus. This was nainly the consequence of the size of the ross sectional area of the fibers in each nuscle. In all three muscles capillary den- ity decreased with increasing body weight lue to the gain in cross sectional area. How- ver, the magnitude of the decrease in capil- ary density could not be entirely accounted or by the changes in area. In fact, in the ibialis anterior, the magnitude of the de- rease was less than expected because of a oncomitant increase in the capillary to iber ratio (C:F) from 1.39 to 1.79, with in- reasing body weight (Table I). This in- rease in C:F appears to be associated with

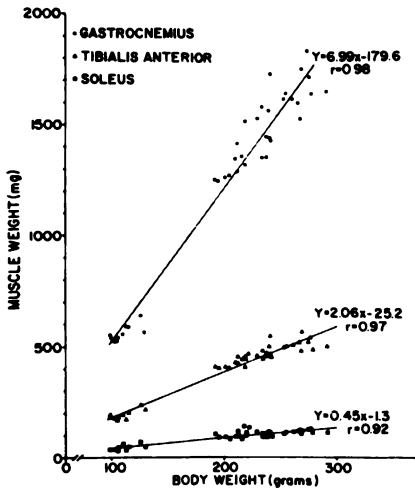

FIG. 2. Relationship between body weight and veight of three muscles of the rat. The gastrocnemius ıcludes the plantaris.

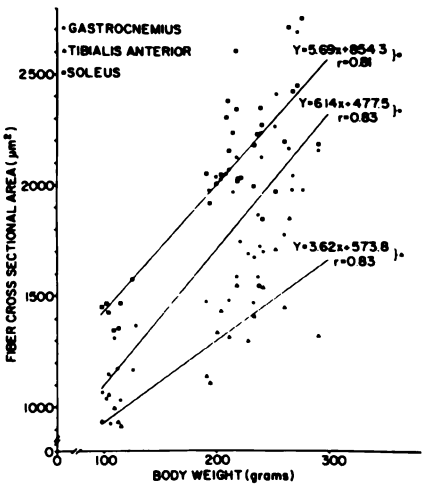

FIG. 3. Relationship between body weight and average cross sectional area of the fibers in three muscles of the rat. These three lines are statistically different; furthermore, the slope of the tibialis anterior line is different from the other two.

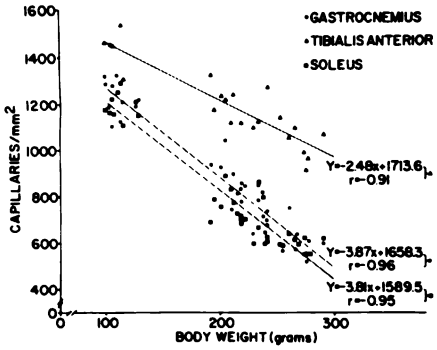

FIG. 4. Relationship between body weight and capillary density in three muscles of the rat. The three lines are statistically different. The slope of the line of the tibialis anterior is different than the other two.

the increase in the relative population of red fibers (Fig. 5). Conversely, in the soleus and the gastrocnemius, the magnitude of the decrease in capillary density was larger than expected on the basis of changes in cross sectional area. In the soleus this was partly

TABLE I. RELATIONSHIPS BETWEEN C:F RATIO AND BODY WEIGHT (GRAMS) FOR THE TIBIALIS ANTERIOR AND SOLEUS MUSCLES OF RATS.

Muscle	Equation	*r*	*P*
Tibialis anterior	C:F = 0.001946 BW + 1.20	0.54	<0.05
Soleus	C:F = −0.001697 BW + 1.93	−0.48	<0.01

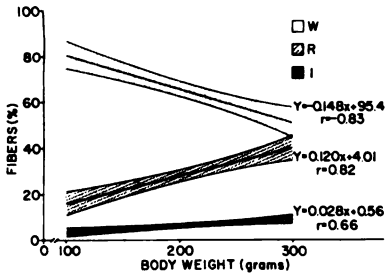

FIG. 5. Average muscle fiber composition in the tibialis anterior in rats of different body sizes. The 95% confidence intervals are shown. A decrease in white fiber population (W) and an increase in red fiber population (R) occurred with maturation ($P < 0.001$). The intermediate fiber population (I) changed only slightly but it was also significant ($P < 0.005$).

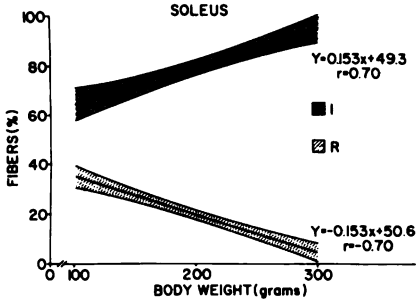

FIG. 6. Average fiber composition and 95% confidence intervals in the soleus. Significant changes in intermediate (I) and red (R) fiber populations were observed with maturation ($P < 0.001$).

due to a decrease in the C:F ratio from 1.76 to 1.42 (Table I), but in the gastrocnemius, the changes in C:F were statistically insignificant. The changes in C:F ratios with increasing body weight were accompanied by changes in fiber composition in the soleus (Fig. 6) while the gastrocnemius exhibited only moderate changes but with less scatter (Fig. 7).

Discussion. It is known that fixation of skeletal muscle with formalin leads to a reduction of approximately 34-56% in cross sectional area (4, 10). We have used frozen muscle samples to measure fiber size and capillary density which minimizes the error due to shrinkage (11) and renders more accurate data. Our capillary density values and fiber dimensions are, therefore, not comparable to most data published in the literature unless the shrinkage factor is *known.*

The rats we studied weighed between 99

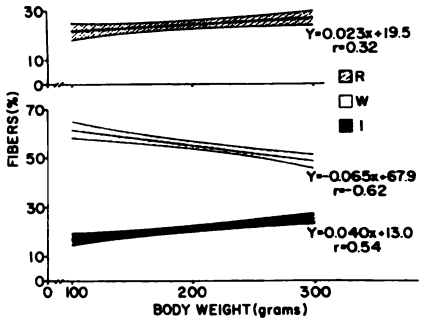

FIG. 7. Average fiber composition and 95% confidence intervals in the medial head of the gastrocnemius. The red fiber population (R) did not change with increasing body weight (upper panel) ($P > 0.05$). However, white (W) and intermediate (I) fibers changed significantly with maturation ($P < 0.001$) (lower panel).

and 291 g, which corresponds to ages of about 35-100 days according to growth charts provided by Simonsen Labs., Inc. This is the period when rats grow rapidly. After 110 days of age these rats grow at a much slower rate. Sprague-Dawley rats from different laboratories have different growth patterns. Our findings, therefore, apply to a specific age and weight group. Rats of these sizes are commonly used in the laboratory.

We have shown that, in young rats, growth causes a predictable increase in muscle weight, mainly due to an increase in the girth of the muscle fibers which is accompanied by a change in fiber composition. Due mainly to this increase in cross sectional area of the fibers capillary density decreases. Valdivia recognized that capillary counts in skeletal muscle of guinea pigs vary with body weight but he did not provide a quantitative estimate of these differences (2). We believe the variability in capillary density with body weight explains in part the wide discrepancies found in the literature (12). Furthermore, we found considerable differences in capillary density, fiber size and composition between muscles of the same animal, an observation previously made by others (11, 13). This probably reflects differences in muscle function.

Our findings strongly suggest that body weight must be considered when capillary density and muscle fiber size and composition are studied in rats and others rodents as

this change represents a decrease in the capacity for O_2 metabolism in older animals. We found a similar decrease in red fiber population in the soleus. For each muscle our data on changes in fiber composition with maturation parallels our findings on C:F ratios. Differences between individual muscles were found. Increases in the percentage of red fibers, that is, in fibers with greater numbers of capillaries around them (7, 11, 14), as seen in the tibialis anterior were accompanied by increases in the C:F ratio, whereas decreases in the percentage of red fibers, as in the soleus, were accompanied by a decrease in the C:F ratio. The explanation for these changes in fiber composition is not yet clear but they probably reflect differences in the physiology of these muscles with age. The fact that changes in fiber composition of skeletal muscle occur with maturation, independently of experimentally imposed conditions, must be born in mind by those interested in quantitating histochemical and structural changes in muscle.

Summary. Capillary density, fiber size and composition, were quantitated by histochemical methods in the tibialis anterior, soleus and gastrocnemius (medial head) of rats of different body weights. The capillary density was different in these muscles and it decreased with increasing body weight in a linear fashion due to a concomitant increase in the average cross sectional area of the fibers. This observation suggests that part of the variability in capillary density found in the literature is due to differences in body size. A change in muscle fiber composition with maturation was also observed. These were more marked in the tibialis anterior and in the soleus than in the gastrocnemius.

The authors gratefully acknowledge the assistance of Ms. Sheila H. Eby.

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Received December 20, 1976. P.S.E.B.M. 1977. Vol. 154.

. OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 467-470 (1977)

ts of Glucosamine, Dibutyryl Cyclic AMP and Lymphocytes on Retrobulbar Fibroblast Synthesis of Hyaluronic Acid (39695)[1]

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interaction of cultured human retro- fibroblasts with lymphocytes has ɜed as an *in vitro* analogy to Graves' lmopathy (1-3). Previously we have ɜtrated that cyclic 3',5'-adenosine hosphate (cAMP) mediates stimula- hyaluronic acid (HA) synthesis in the ɜsts induced by lymphocytes. We ow that glucosamine (GlcN), a pre- of HA, enters the fibroblasts and is ɔrated into HA. The use of GlcN on tures facilitated investigation of loci biosynthetic process at which the could act. Studies here reported ıat the intracellular concentration of ɜ rate limiting in fibroblast synthesis , and that lymphocytes and dibutyryl (DbcAMP) appear to stimulate HA ıis at a point which is dependent upon ned GlcN.

rials and methods. Cultures of fibro- rom normal human retrobulbar con- tissue were initiated and perpetu- described previously (2). Lympho- rom normal subjects were obtained ɔzen-thawed before use by a method n a prior publication (1). D-Glucosa- lCl, D-galactosamine-HCl (GalN), ıcosamine-6-PO_4 as the sodium salt 6-PO_4) were purchased from the Chemical Company (St. Louis, Mo.);)-dibutyryl adenosine 3',5'-cyclic ate (DbcAMP) was obtained from z/Mann (Orangeburg, N.Y.), D-[1- cosamine-HCl ([^{14}C]GlcN), 9.6 mCi/ was purchased from New England r Corporation (Boston, Mass.). The above materials were dissolved in CMRL 1066 medium and sterilized by filtration.

Medium composition was as recorded before (2), and experimental design, except where noted, was as previously described (4). Methods for analyzing media glucose, media glycosaminoglycans (assumed to be HA from prior studies), and fibroblast protein have been reported (2).

[^{14}C]GlcN was added to cultures in the quantity of 0.9 μCi and 92 nmole per flask. The [^{14}C]HA was determined by dissolving the cetylpyridinium-HA precipitate (obtained in the extraction GAG from media for uronic acid analysis) in methanol and counting the ^{14}C in a liquid scintillation counter. The ^{14}C background counts which relate to contaminating [^{14}C]GlcN were obtained by adding the 0.9 μCi of [^{14}C]GlcN to flasks in each experimental group at the end of the culture period; this background activity (5% or less of the total ^{14}C count rate) was subtracted to give the net [^{14}C]HA activity. The net [^{14}C]HA was divided by the total [^{14}C]GlcN activity added to give the fraction of medium GlcN converted to HA. The total HA derived from medium GlcN was then calculated by multiplying the fraction converted by the nanomoles of GlcN present in the medium.

Results. Additions of GlcN, 5×10^{-4}–5×10^{-3} *M*, to the media of fibroblast cultures resulted in dose-related increases in HA synthesis. However, no additional HA production was observed when GlcN concentrations were increased above 5×10^{-3} *M*; indeed, the quantities of HA recovered in these circumstances were often less (Tables I and II). Glucose utilization was inconsistently affected, and fibroblast protein of each flask was unaltered by the presence of GlcN.

Glucosamine-6-PO_4, the intracellular form of GlcN entering the HA biosynthetic

study was carried out under the auspices of tates Public Health Service Grant No. AM 'resented in part at the Central Society for Research in Chicago, November 8, 1975, and ; in abstract form in Clin. Res. **23**, 517A

1977 *by the Society for Experimental Biology and Medicine* erved.

ISSN 0037-9727

pathway, was no more effective and, at times, less effective than GlcN in enhancing HA synthesis. Additions of GalN, 10^{-4}–10^{-2} *M*, did not alter HA production. Glucose enrichment of media up to 5×10^{-3} *M* resulted in little or no increases in HA production.

Stimulations of fibroblasts by DbcAMP in the presence of GlcN, 5×10^{-3} *M*, produced quantities of HA which were greater than those calculated by summing the HA responses observed when DbcAMP and GlcN were added independently to cultures (Table II). Concentrations of GlcN down to 10^{-4} *M* potentiated the DbcAMP effect. However, increasing the concentrations of GlcN to 10^{-2} *M* in association with DbcAMP resulted in no additional synthesis of HA. Comparable effects of GlcN on the synthesis of HA were seen in experiments where lymphocytes replaced DbcAMP (Table II).

To evaluate the importance of intracellular GlcN in the response to DbcAMP control medium and medium containing GlcN were removed from the cultures after 2 days and just before the addition of the cyclic nucleotide derivative (Table III). In Group B (Table III), GlcN was introduced into the cultures for only a few minutes before DbcAMP to assess the effect of residual GlcN in the medium, i.e., the GlcN which could not be removed in the change of media. Residual GlcN enhanced the stimulation of HA synthesis by DbcAMP (Group B compared with Group A). However, when the fibroblasts were exposed to medium enriched with GlcN for 2 days before the addition of DbcAMP, the production of HA was significantly greater than that obtained when GlcN had been present only briefly (Group C compared with Group B in Student's *t* test).

By following the fate of radiolabeled GlcN, the GlcN in medium was observed to be readily incorporated into HA (Table IV). In the presence of medium enriched with GlcN (Group B in Table IV), most, if not all, of the HA was composed of GlcN which

TABLE I. EFFECTS OF GLUCOSAMINE ON RETROBULBAR FIBROBLAST SYNTHESIS OF HA.

Concentration of GlcN ($\times 10^{-3}$ *M*)	No. of flasks	HA in media[a] (nmole of uronic acid/mg fibroblast protein)[b]	(E/C)[c]
0.0	(4)	71 ± 4	
0.5	(4)	124 ± 10	1.75
1.0	(4)	149 ± 7	2.10
5.0	(4)	180 ± 17	2.54

[a] Media collected over Days 2–4 of culture.
[b] Mean ± SEM.
[c] Experimental group mean ÷ control group mean.

TABLE II. EFFECTS OF GLUCOSAMINE ON THE STIMULATION OF RETROBULBAR FIBROBLAST PRODUCTION OF GAG BY DIBUTYRYL CYCLIC AMP AND LYMPHOCYTES.

Additives and concentrations		No. of flasks	HA in media[a] (nmole of uronic acid/mg of fibroblast protein)[b]	(increments above basal concentration)
Experiment No. 1				
None		(4)	151 ± 5	
DbcAMP	2×10^{-3} *M*	(3)	587 ± 15	436
GlcN	5×10^{-3} *M*	(4)	264 ± 4	113
GlcN	10^{-2} *M*	(4)	245 ± 7	94
DbcAMP plus GlcN	2×10^{-3} *M* + 5×10^{-3} *M*	(4)	1034 ± 26	883
DbcAMP plus GlcN	2×10^{-3} *M* + 10^{-2} *M*	(3)	995 ± 63	844
Experiment No. 2				
None		(4)	61 ± 5	
Lymphocytes	5×10^{5}/ml	(4)	167 ± 6	106
GlcN	5×10^{-3} *M*	(4)	240 ± 14	179
GlcN	10^{-2} *M*	(4)	231 ± 11	170
Lymphocytes plus GlcN	5×10^{5}/ml + 5×10^{-3} *M*	(4)	479 ± 23	418
Lymphocytes plus GlcN	5×10^{5}/ml + 10^{-2} *M*	(4)	389 ± 21	328

[a] Media collected over Days 2–4 of culture.
[b] *Mean ± SEM.*

ted external to the cells. Medium
at 5×10^{-3} *M*, also served as the sole
of this precursor when HA synthesis
mulated by DbcAMP (Group D in
IV). The HA calculated (by ^{14}C
ıg) to be derived from medium GlcN
up D (269 nmole) seems to exceed
al HA measured as uronic acid (231
, but the two values are not signifi-
different by Student's *t* test.

ıssion. In the presence of media en-
with GlcN, there is enhanced synthe-
HA production in retrobulbar fibro-
ultures stimulated by lymphocytes
DbcAMP (which is considered to act
s the intracellular mediator, cAMP,
lymphocytes' action). Potentiation
ıave related to the effect of cAMP on
an expanded intracellular pool of GlcN or to cellular uptake of GlcN which was accelerated by cAMP. In Table III, greater potentiation was seen when the fibroblasts were exposed to GlcN for 2 days before the introduction of DbcAMP in new media (Group C) than when GlcN was presented to the cells for only a few minutes (Group B): these data indicate that at least some of the potentiation of the DbcAMP stimulation resulted from GlcN which had already entered the fibroblasts.

There was some potentiation of the response to DbcAMP in Group B in Table III which must have been related to residual GlcN not removed from the culture flasks when the media were changed. This residual GlcN could have been taken up by the fibro-

.E III. Effects on Retrobulbar Fibroblast Production of GAG when Glucosamine is Added to and Removed from Media.

Additives and concentrations		No. of flasks	HA in media (nmole of uronic acid/mg of fibroblast protein)[b]	(increment above respective basal concentrations[c])
Days 2-3[a]	Days 3-5			
None	None	(5)	43 ± 1	356
None	DbcAMP, 2×10^{-3} *M*	(5)	399 ± 11	
None	GlcN, 5×10^{-3} *M* briefly, then none	(5)	47 ± 2	416
None	GlcN, 5×10^{-3} *M* briefly, then DbcAMP, 2×10^{-3} *M*	(5)	463 ± 33	
GlcN, 5×10^{-3} *M*	None	(5)	78 ± 3	499
GlcN, 5×10^{-3} *M*	DbcAMP, 2×10^{-3} *M*	(5)	577 ± 28	
GlcN, 5×10^{-3} *M*	GlcN, 5×10^{-3} *M*	(5)	135 ± 4	

:r Day 3, media were removed and flasks were washed with 1 ml of respective medium to be used on Days
n medium for Days 3-5 added. In group B, GlcN, 5×10^{-3} *M*, 2.5 ml, was added before wash and
tely removed.
ın ± SEM.
ach group, mean of Subgroup (1) subtracted from mean of Subgroup (2).

LE IV. Influence and Fate of Media Glucosamine in the Synthesis of Hyaluronic Acid by Retrobulbar Fibroblasts.

Additives to media	Hyaluronic acid synthesized[a] (nmole of uronic acid/flask)		(nmole derived from GlcN in media)[b]	
	No.[c]	Mean ± SEM	No.[c]	Mean ± SEM
	(4)	26 ± 2	(4)	0.62 ± 0.06
, 5×10^{-3} *M*	(4)	55 ± 6	(4)	43 ± 12
ıMP, 10^{-3} *M*	(4)	87 ± 2	(4)	3.5 ± 0.5
, 5×10^{-3} *M* plus DbcAMP 10^{-3} *M*	(4)	231 ± 3	(4)	269 ± 31

ia collected over Days 2-4 of culture.
ulated by recording the percent of [^{14}C]GlcN incorporated into HA and relating this value to the total
nitial media: Groups A and C had 92 nmole of GlcN/flask (from the commercial [^{14}C]GlcN), and Groups
had 12,592 nmole of GlcN/flask.
ia obtained from parallel flasks for each assay.

blasts through a cAMP-dependent or a cAMP-independent process. Thus, although DbcAMP, and presumably cAMP, can be shown to act on intracellular GlcN or on a site dependent upon intracellular GlcN, the possibility that the cyclic nucleotide also enhances cellular entry of GlcN cannot be excluded.

In HA synthesis, preformed GlcN appears to be preferentially utilized over GlcN converted from glucose by the fibroblasts (Table IV). Thus, the availability of GlcN seems to be one rate-limiting step in the synthesis of HA. However, since medium concentrations of GlcN greater than 5×10^{-3} *M* induce no additional effect, but rates of HA synthesis can be further enhanced by DbcAMP, there also appears to be a second, cAMP-dependent, rate-limiting step. This second step is dependent upon, but subsequent to, the availability of intracellular GlcN.

There are alternative explanations of the observed data. It is possible that the system which transports GlcN into the cell becomes saturated when medium concentrations of GlcN reach 5×10^{-3} *M*. Although at least some effects of DbcAMP relate to the intracellular GlcN, an action of the cyclic nucleotide derivative which enhances the entry of GlcN into fibroblasts, even after the normal transport system has been saturated, is possible. In this case, no rate-limiting step subsequent to the formation of GlcN and dependent upon cAMP needs to be postulated.

Another mechanism may contribute to the results of the experiments. Glucosamine, at concentrations above 2×10^{-3} *M*, has been shown to inhibit glycolytic enzymes and to reduce the concentration of ATP in retinas (5). Therefore, the failure of concentrations of GlcN above 5×10^{-3} *M* to increase HA synthesis by retrobulbar fibroblasts may relate to toxic effects attributable to GlcN. The toxic effects may also have limited the synthesis of endogenous cAMP,

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 471–474 (1977)

Fluctuations of Plasma Cholesterol in the Female Miniature Swine and Its Relationship to Progesterone Secretion (39696)[1]

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have been few studies on the influ- he estrous cycle on blood lipid con- ons in animals. In one report, the asma cholesterol measured during strous cycle was found to be higher proestrus and estrus than during (1). In another study (2), blood was l from mature ewes on the day of ad 6 days later during diestrus. Se- lesterol concentrations tended to be uring diestrus, but the difference be- ne two phases was not statistically nt. To our knowledge this informa- not available for porcine animals, e often used as models for the study osclerosis.

g our studies on the effect of porta- astomosis on cholesterol metabo- he female miniature swine, we were ed in some animals with the prob- xcessive variation in plasma choles- hanges as large as 50% were ob- or some animals during the preoper- ase, with values in the range of 130- /dl, considered inordinately high d to the 80–100 mg/dl usually nd expected for normal pigs in the ge studied (3). This phenomenon even more serious when it reap- in the postoperative phase since it / compromised evaluation of the ef- he shunt on plasma lipids. Daily or blood sampling over long periods that the fluctuation was cyclic and o suspect an effect of the estrous hich was confirmed in the results to ted.

ials and methods. Six noncastrated female miniature pigs (Sinclair Research Farm, University of Missouri, Columbia, Missouri) aged 8–10 months were housed indoors and fed a standard pig grower mash. Four animals underwent a portacaval anastomosis, whereas two were sham-operated with surgical manipulations which included vascular clamping.

Total plasma cholesterol and progesterone were measured on samples obtained in the morning during long-term studies started 5 weeks after the operations and continuing over a total of 13 presumptive estrous cycles. Cholesterol was measured on a Technicon Autoanalyzer by the method of Block *et al.* (4). Progesterone was determined by radioimmunoassay by the method of DeVilla *et al.* (5). The coefficient of variation for our method for cholesterol assay (replicate determinations) is less than 3%.

Results. The plasma progesterone concentration was only 0.1–1.5 ng/ml during an estrogenic phase averaging 7 days, then rose rapidly to high values (15–50 ng/ml) and declined again over the next 14 days. This indicated an estrous cycle of 21 days, in agreement with previous reports (6, 7). All six animals demonstrated ovulatory cycles (Fig. 1). Plasma cholesterol varied inversely with progesterone in five of the six animals (Fig. 1). High cholesterol levels were evident for 3–6 days during the follicular phase; the peak value was usually attained 2 or 3 days before the estimated ovulation day. The latter was assumed to correspond to or immediately follow the rise in plasma progesterone. The sixth (sham-operated) animal showed normal progesterone secretion but gave no predictable cyclic pattern for cholesterol. This animal had had one anovulatory cycle prior to surgery.

The average increase in cholesterol con-

rted by grants from the Macdonald-Stewart n, the Medical Research Council of Canada '), and the C.O. Monat Foundation.

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ISSN 0037-977

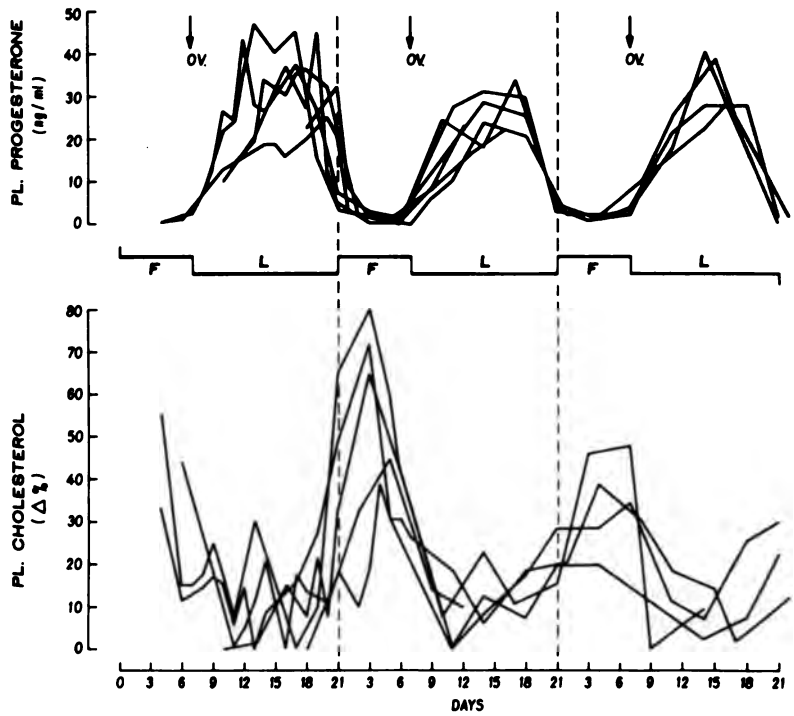

FIG. 1. Inverse cyclic relationship between plasma concentrations of progesterone (ng/ml) and chole (percentage of increases over the lowest value in the luteal phase) during 21-day estrous cycles. F and L re follicular and luteal phases, OV. to approximate time of ovulation estimated from time of onset of the incre plasma progesterone.

centrations at the peak was 50% of the minimum value during the progesterone secretion period. The cyclic changes persisted throughout the study and were similar for shunted and sham-operated animals (Fig. 2). However, the variations in plasma cholesterol were of a lesser amplitude in one shunted animal (No. 26, Fig. 2), whose condition deteriorated 4 months after surgery (extensive liver damage being noted at autopsy), while the progesterone secretory pattern remained unchanged.

To document this investigation further, progesterone was also measured in five plasma samples obtained from three different animals preoperatively, for which inappropriately high levels of cholesterol were observed. Progesterone levels were found to be low in every case (0.2–1.1 ng/ml).

Discussion. We do not know how sex hormones influence plasma cholesterol in the miniature swine. In their study of cholesterol and bile acid turnover in female miniature swine, Dupont *et al.* (8) found great

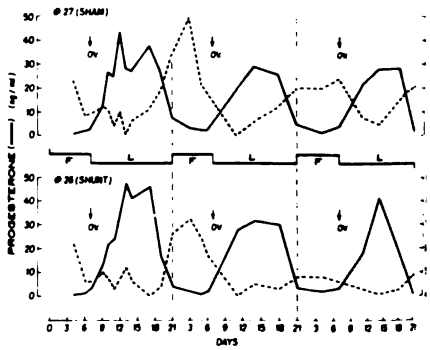

FIG. 2. Similar cyclic fluctuations for progest and cholesterol in the plasma of sham-operated (al and shunted (below) single miniature swine. F, L OV. as in Fig. 1.

variation from sample to sample but thou this might be attributed to frequent s pling and to the effect of digestion, since bleeding was done postprandially. W and Belbeck (9) found that the turnove triglyceride fatty acids was increased i female swine which was reportedly in h

firmed by serum estrogen assay. Their did not refer to plasma cholesterol.
eral blood lipid concentrations have shown to be affected during the hor- changes occurring during the human rual cycle, but observations have been le. Some authors have reported an se in cholesterol at the time of ovula- .0, 1), others (11) a decrease, whereas ne studies no differences were found 3). Whenever changes were observed, were usually small and, thus, could y be missed unless blood was sampled In one study (14), lipid concentra- were measured simultaneously with y excretion of estrogens, pregnane- and several other steroids. While no ation was found between plasma cho- ol and urinary hormone concentra- an increase in total cholesterol during llicular phase and a decrease during teal period were observed. Simultane- eterminations of plasma cholesterol asma gonadal steroids have so far not eported in humans.

ile increases in plasma cholesterol and ally triglycerides are well documented nan pregnancy (15, 16), alterations of a lipids in pregnant animals vary y according to species. Increased cho- ol levels occur in the rat (17) and the 18), whereas, in the rabbit (19) and esus monkey (20), a continuous drop sma cholesterol is observed as preg- progresses. There has been, so far, no on this phenomenon in the swine.

results show an evident inverse rela- ip between total plasma cholesterol rogesterone levels. The cholesterol attained during the follicular part of cle are often sharp and short-lived and t correspond to the estimated day of ion. Thus, random occasional blood ing could easily cause these changes to noticed. To our knowledge, this is the eport of a cholesterol fluctuation pat- l on the estrous cycle in the swine. Its is such that blood sampling 2-3 days ovulation may yield, in extreme plasma cholesterol values 80% higher hose obtained during the luteal phase l).

iew of the growing popularity of mini- ature swine as an experimental model for the study of lipid and cholesterol metabolism (8, 9, 21-23) and because of the frequent choice of females because of their mild disposition, one should be aware of this physiological phenomenon. Failure to take it into account could seriously compromise the results of such experiments and lead to incorrect interpretation.

Summary. Large fluctuations in plasma cholesterol concentration were noted during a study on the metabolic effects of portacaval anastomosis in the female miniature swine. The fluctuations were cyclic and related to the estrous cycle, as shown by measurements of total plasma cholesterol and progesterone on samples obtained almost daily from six animals over several estrous cycles. Hormone concentrations indicated a 21-day estrous cycle consisting of a 7-day follicular phase and a 14-day luteal period. Plasma cholesterol fluctuated in a cycle which was the inverse of that for progesterone: High cholesterol concentrations were observed for 3-6 days during the follicular phase with peak values as much as 80%, and on the average 50%, higher than the mean levels observed during the luteal phase. Failure to recognize these plasma cholesterol cyclic fluctuations can totally confuse the interpretation of studies on cholesterol metabolism in swine, which are increasingly popular as experimental models of atherosclerosis and lipid metabolism.

We are indebted to Miss Murielle Paquette, Miss Francine Sainte-Marie, and to our animal-care staff for their expert technical assistance.

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Received October 21, 1976. P.S.E.B.M. 1977, Vol 154.

)F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 475–478 (1977)

mulation of Prolactin Secretion by Metoclopramide in the Rat (39697)[1]

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:lopramide (2-methoxy-5-chloropro-
de) is a procainamide derivative that
n used in Europe for 12 years as
:metic and stimulant of smooth mus-
e upper gastrointestinal tract (1, 2).
g, presently under investigation in
ted States, is a potent stimulant of
ı (PRL) secretion in man (3–5);
mulating doses (10–20 mg) of meto-
nide produce serum drug levels of
00–200 ng/ml in humans (6). Al-
:linical evidence (4, 5) has suggested
ıe antagonism as a mechanism for
pramide-induced PRL secretion,
ıve been no direct tests of this hy-
. In the present study, we describe
ents which lend support to a dopa-
tagonistic action of metoclopramide
oting PRL secretion.

ials and methods. In vivo experi-
Groups of six 200-g male Sprague-
rats received intraperitoneal injec-
: 100 μg of metoclopramide dis-
ın 0.5 ml of physiologic saline or
lone. Sixty minutes after injection,
were killed by decapitation, and the
)lood was collected for hormonal
.

ro experiments. Hormone secretion
)lated rat pituitary tissue was studied
ple perifusion system previously de-
(7). In brief, two pituitary quarters,
d from a 150–250-g male Sprague-
rat, were placed on a wire grid in a
; steel Millipore-Swinny filtration
r and were immersed in a 37° water
lodified Gey and Gey buffer (7),
ated with 95% O_2–5% CO_2, was
l through the chamber, and, after a
preincubation period, the effluent
was collected in 2-ml (4 min) aliquots on a fraction collector for hormonal assay. Test substances dissolved in Gey and Gey buffer were introduced by means of a three-way stopcock. Mean effluent hormone levels during the final 20 min of the initial control period were compared with mean levels during the subsequent period of administration of test substances. At least three experiments using pituitaries from separate animals were performed for each concentration of test substance. Student's *t*-tests for paired or unpaired data were used, as appropriate, to assess the significance of observed changes in hormone levels.

Hormone assays and reagents. Rat serum and perifusion effluent PRL and growth hormone (GH) concentrations were measured using specific radioimmunoassays (7, 8). Reagents for both assays were supplied by Dr. A. F. Parlow, NIAMDD Hormone Distribution Program. The rat PRL standard used for radioimmunoassay (NIAMDD Rat PRL RP-1) had a bioassay potency of 11 IU/mg, whereas the GH standard (NIAMDD Rat GH RP-1) had a potency of 0.6 IU/mg. Crystalline metoclopramide was kindly supplied by the A. H. Robins Co., Richmond, Va., and dopamine hydrochloride was purchased from Sigma Chemical Company.

Results. In vivo experiments. Intraperitoneal administration of 100 μg of metoclopramide resulted in a significant ($P < 0.02$) increase in serum PRL levels 60 min later; the mean serum PRL was 85 ± 14 (SEM) ng/ml in the metoclopramide-treated rats compared to 31 ± 10 ng/ml in the saline-treated controls. Serum GH, in contrast, was not significantly affected; the mean serum GH following metoclopramide treatment, 39 ± 22 ng/ml, was similar to the value of 35 ± 16 ng/ml observed in saline-treated controls.

ırted by the Medical Research Service of the Administration and by funds from the A. H.)mpany, Richmond, Virginia.

1977 by the Society for Experimental Biology and Medicine
rved.

ISSN 0037-9727

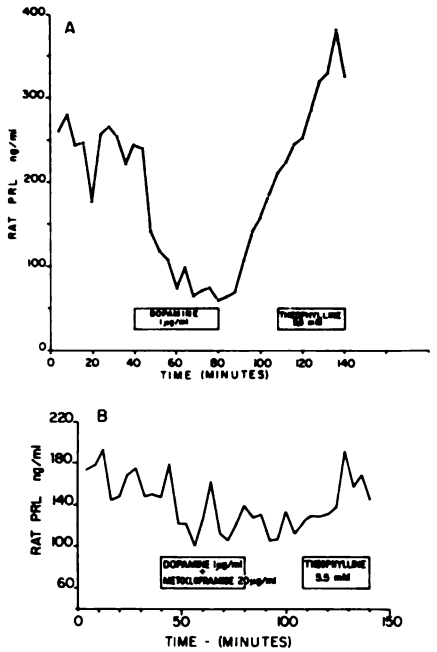
In vitro experiments. Basal perifusion effluent hormone levels varied from experiment to experiment, depending on the size of the pituitary glands used. Perifusion experiments failed to demonstrate any direct effect of metoclopramide on PRL secretion over the concentration range of 0.2–200 μg/ml (Table I); Fig. 1 illustrates this lack of effect for perifusate metoclopramide concentrations of 20 μg/ml. Only a slight downward drift in perifusion effluent PRL levels, also seen during control periods, was observed during metoclopramide perifusion. In contrast, 5.5 m*M* theophylline regularly provoked PRL release (Fig. 1), demonstrating tissue responsiveness. GH responses to perifused metoclopramide were variable; while metoclopramide concentrations of 200 ng/ml produced no significant effect on GH levels, 20 μg/ml concentrations of the drug significantly ($P < 0.05$) lowered effluent GH concentrations.

Dopamine (1 μg/ml of dopamine hydrochloride = 5.3×10^{-6} *M*) produced prompt and significant ($P < 0.01$) inhibition of PRL secretion from the perifused pituitary tissue (Fig. 2A and Table I); mean effluent PRL levels during dopamine administration were 40% of preceding control values. In the presence of metoclopramide (20 μg/ml), however, this inhibitory effect of dopamine was markedly blunted; although a small decrease in perifusate PRL levels occurred, this was indistinguishable from the slight fall observed with metoclopramide alone ($P > 0.2$; see Fig. 2B and Table I). Dopamine also produced a significant fall in perifusate GH levels; this effect was similarly blocked by metoclopramide (Table I).

TABLE I. EFFECT OF METOCLOPRAMIDE ON BASAL A
PERFUSED R

Drug	Perifu Prolact Control
Metoclopramide (0.20 μg/ml)	55.8 ± 15.9 (6)[a]
Metoclopramide (20 μg/ml)	63.2 ± 14.9 (5)
Metoclopramide (200 μg/ml)	37.6 ± 7.3 (3)
Dopamine-HCl (1 μg/ml)	216 ± 19.2 (4)
Dopamine-HCl (1 μg/ml) + Metoclopramide (20 μg/ml)	142 ± 13.2 (4)

[a] Numbers within parenthesis are number of experiments at each conc
[b] Significantly different from corresponding control period, $P < 0.05$.
[c] Significantly different from corresponding control period, $P < 0.01$.



G. 2. (A) Inhibitory effect of dopamine (1 μg/ on prolactin release from perifused rat hemipitui- (B) Blunting of prolactin inhibitory effect of do- ne (1 μg/ml) in the presence of metoclopramide μg/ml). For both A and B, typical perifusion ex- nents are shown.

ems of the rat and cat (15), providing her support for a dopamine-antagonistic on of the drug. In either case, we have id no evidence for a direct stimulatory ct of metoclopramide on PRL release; lable data therefore suggest that meto- ramide elevates serum PRL *in vivo* by icing dopamine-mediated inhibition of actin secretion. Such a mechanism ld also be consistent with observations lunted PRL responses to metoclopram- in humans pretreated with L-DOPA (4, Although our data support a pituitary of dopamine antagonism, they do not out an additional or alternative hypo- amic site of action. The clinical observa- of extrapyramidal side effects in pa- ts receiving large doses of metoclopram- chronically (16, 17) supports a possible for metoclopramide as a CNS antago- of dopamine, since depletion of dopa- e in the basal ganglia is thought to occur 'arkinson's disease (18).

uijada *et al.* (19) have previously reported a modest inhibition by dopamine of rat GH secretion *in vitro*; our observations confirm these findings and further demonstrate that this effect, like the PRL-inhibiting actions of dopamine, is blocked by metoclopramide.

Summary. Metoclopramide, a procainamide derivative known to raise serum prolactin (PRL) levels in intact humans, produced a significant increase in serum PRL when administered intraperitoneally to male rats. In contrast, direct application of metoclopramide to the isolated rat pituitary in a perifusion system did not increase prolactin release *in vitro*. In this system, dopamine inhibited prolactin secretion. Perifusion with both metoclopramide and dopamine blocked the inhibitory effect of dopamine on prolactin release. This suggests that metoclopramide promotes PRL secretion by antagonism of dopamine-mediated CNS-dependent mechanisms. Although metoclopramide had no effect on serum growth hormone in the intact rat, the drug antagonized the growth hormone-inhibiting action of dopamine *in vitro*.

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Received October 27, 1976. P.S.E.B.M. 1977, Vol. 154.

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ıanical Properties of Cardiac Muscle from Spontaneously Hypertensive Rats: Accentuated Aftercontractions (39698)

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ɛral studies have indicated that a vari- indices of cardiac contractility and may be elevated in the early phases ɛrtension in both man and experimen- mals (1-7). The hyperdynamic state e a result of an expanded blood vol- l), a redistribution of blood resulting xpanded central blood volume (2, 7), ıltered autonomic neural input to the 3, 6, 7). However, the possibility that ions in intrinsic contractile properties muscle itself contribute to the hyper- ıic state has not yet been examined.

spontaneously hypertensive rat ı (8) has been shown to develop se- ustained hypertension which is ini- ssociated with an elevation in cardiac (5). In this study, isometric proper- papillary msucle preparations were ıed to ascertain whether differences hanical properties between SHR and tensive Wistar-Kyoto control rats) do exist.

ıods. Male albino spontaneously hy- sive rats (SHR) were compared to ıtched normotensive Wistar-Kyoto l animals (WKY).

mals were anesthetized with sodium ɔarbitol, the carotid artery was cannu- and blood pressure was measured us- ɔtatham P23Db transducer connected ɔrush 440 recorder. The thorax was d, and the heart was rapidly removed aced in oxygenated physiological salt ɔn containing: NaCl, 115 m*M*; KCL, *M*; $MgCl_2$ and $CaCl_2$, 2.5 m*M*, glu- l1.5 m*M*; Tris-Cl, 35.0 m*M*; pH 7.4. ɾing removal of a left ventricular pap- muscle, the heart was weighed. The ɛ was mounted in a muscle bath ɔed with (i) a water jacket for temper- ɛontrol at 27 ± 0.5°, (ii) a gas portal ivery of 100% O_2, and (iii) inflow and ɯ portals for circulation of the solu- A 27-mm artery clamp was rigidly mounted in the bottom for attachment of one end of the muscle. An identical top clamp was attached to a silver chain connected to a moveable Statham isometric transducer (green cell). Changes in muscle length could be made and measured to a precision of ±0.01 mm. Output of the force transducer was amplified by a Gould transducer coupler (Type 114307 04) and was recorded on a Gould strip chart Model 440. The force signal was differentiated by a Gould differentiator coupler (Type 134214 01) and was also recorded.

The stimuli were provided by a Grass stimulator Model S88 and were applied through a pair of stainless steel electrodes positioned approximately 5 mm on either side of the muscle. Voltage and duration of the stimulus were adjusted to just elicit maximal mechanical responses from each preparation and, thus, were well below those levels required to release endogenous catacholamines (9).

After the muscle was mounted in the bath, it was stimulated at 0.2 Hz with a resting tension of 1-2 g for about 1 hr during which time the active tension reached a steady state.

(i) *Twitch characteristics at* L_{max}. The muscle was stretched in 0.5-mm increments to determine the length at which isometric tension development was maximal (L_{max}). At this length, resting and active tension, time-to-peak tension development (TPT), rate of tension development (dT/dt), and $^1/_2$ relaxation time ($^1/_2$ RT) were determined. Since this determination was made during the initial stretching sequence of length changes and muscle length-tension curves show considerable hysteresis (10), resting tensions are high. With a subsequent release of tension to 1.0 g, active tension did not decrease noticeably. At the end of the experiment, the muscle was removed and weighed. The cross-sectional area was esti-

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ISSN 0037-9

mated assuming a tissue density of 1.0 and a cylindrical muscle strip. All tension data were expressed per millimeter squared.

(ii) *Mechanical refractory period determined by paired pulses (MRP).* Following measurement of the twitch characteristics at L_{max}, muscle length was adjusted to that which gave a resting tension of 1.0 g. After equilibrating at 0.2 Hz at this new length, paired stimuli were presented. The pattern of presentation consisted of alternating four single stimuli with four paired stimuli with the overall frequency remaining at 0.2 Hz. The delay between the paired stimuli was progressively increased from 60 to 1500 msec in steps ranging from 25 to 500 msec. The paired pulse experiment was then repeated at a faster frequency of stimulation (0.5 Hz). The delay at which a response first appeared to the second stimulus of the pair was designated as the mechanical refractory period (MRP). As the delay was further increased, amplitudes of the individual responses to the two stimuli of the pair were measured. Aftercontractions (Fig. 1) were noted in many preparations following the paired pulse stimulation and were recorded as a percentage of the amplitude of the response to the first stimulus of the pair.

Data analysis. Data were averaged and reported as mean ± standard error of the mean. Differences between SHR and WKY groups were assessed by applying Student's *t*-test.

Results. SHRs had significantly higher arterial pressure when compared with age-matched normotensive WKYs as shown in Table I. Heart weights were somewhat

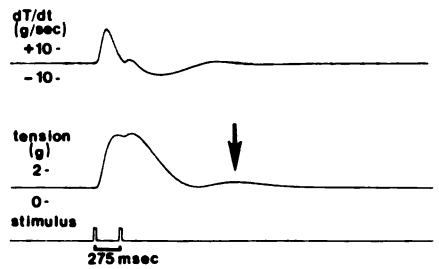

FIG. 1. Isometric response to paired pulse stimuli and subsequent aftercontraction (arrow) in a papillary muscle preparation from a spontaneously hypertensive *rat.*

TABLE I. INFORMATION ABOUT SPONTANEOUSLY HYPERTENSIVE RATS (SHR) AND NORMOTENSIVE WISTAR-KYOTO CONTROL RATS (WKY) USED IN THE EXPERIMENT

	SHR (N = 13)		WKY (N = 13)
Age (days)	84 ± 4[a]		81 ± 4
Body weight (g)	274 ± 22		290 ± 20
Heart weight (g)	0.93 ± 0.03		0.82 ± 0.07
H.W./B.W. × 100	0.33 ± 0.01	*	0.28 ± 0.01
Mean arterial pressure (mm Hg)	189 ± 6	*	120 ± 5

[a] Data reported as mean ± SEM.
* $P < 0.001$.

higher in SHRs than in WKYs as were heart weights expressed as percentage of body weight (H.W./B.W. × 100). However, cross-sectional areas of the SHR papillary muscle (0.94 ± 0.10 mm²) were not different from those of WKYs (0.88 ± 0.09 mm²).

(*A*) *Steady-state responses at L_{max}.* Comparison of isometric properties of the papillary muscles equilibrated at 0.2 Hz at L_{max} revealed no difference in resting tension (2.6 ± 0.3 g/mm² vs 2.6 ± 0.4 g/mm²), active tension (4.9 ± 0.5 g/mm² vs 5.4 ± 0.5 g/mm²), dT/dt (35 ± 4 g/mm²/sec vs 36 ± 4 g/mm²/sec), time-to-peak-tension development (166 ± 3 msec vs 167 ± 2 msec), or ½ relaxation time (176 + 7 msec vs 185 + 6 msec) between SHRs and WKYs, respectively.

(*B*) *Paired pulse responses. (i) Mechanical refractory period.* As shown in Table II, the MRP was found to be significantly longer in SHR preparations than in WKY preparations at both 0.2 and 0.5 Hz stimulation frequencies, even though increasing the frequency of stimulation decreased the MRP in both SHRs and WKYs.

As the delay between paired stimuli increased, the amplitude of the response to the second stimulus increased and, at the maximum delays investigated, approached the amplitude of the initial twitch. At delays below 500 msec, the second response was superimposed on the relaxation phase of the initial twitch, and precise quantitation of the amplitude of the isolated second response was not possible. No differences were detected between SHR and WKY preparations in the relationship between delay and

ABLE II. Isometric Response to Paired timuli: Mechanical Refractory Period[a]

	SHR		WKY
hz (msec)	246 ± 4	← * →	229±6
	↑		↑
	**		**
	↓		↓
hz (msec)	221 ± 4	← * →	205 ± 5

linimal millisecond delay between paired stimuli ill elicit a second response.
< 0.05.
< 0.01.

mplitude of the second response.

Aftercontractions. Aftercontractions induced by paired pulse stimulation in preparations. Table III summarizes characteristics of these contractile s. The incidence and the amplitude of events at both 0.2 and 0.5 Hz were er in SHR than in WKY preparations. n the frequency of stimulation was in- ed from 0.2 to 0.5 Hz, the delay be- n paired stimuli that elicited the largest contractions was decreased signifi- y ($P < 0.01$) in SHR preparations. n aftercontractions did appear in WKY arations at 0.5 Hz, they were much er than at 0.2 Hz ($P < 0.001$).

cussion. Hypertension in SHRs devel- between 4 and 8 weeks of age and is y established by the time the animal is weeks old (11). Cardiac hypertrophy, ver, is evident at even earlier ages suggesting that increased afterload not be the only factor stimulating ab- al growth of the heart. The animals in this study were approximately 12 s of age, and both hypertension and ac hypertrophy were evident.

ce isometric properties of papillary les from SHRs equilibrated at L_{max} are ifferent from WKYs, the tissue's maxi- tension-generating capabilities are ap- tly not influenced at this time by the rtension or hypertrophy. These find- imply that the slow-developing cardiac rtrophy seen in SHRs may have more e characteristics of volume-induced hy- ophy, which does not depress contrac- roperties of isolated muscle (13), than ressure-induced hypertrophy, which depress these properties (14, 15). This stion is supported by studies of Pfeffer

TABLE III. Isometric Response to Paired Pulse Stimulation: Characteristics of Aftercontractions[a]

	SHR		WKY
At 0.2 Hz			
Incidence	13/13		10/13
Optimal delay (msec)[b]	280 ± 8	*	265 ± 8
Amplitude (%)[c]	4.2 ± 0.5	**	2.6 ± 0.5
At 0.5 Hz			
Incidence	13/13		6/13
Optimal delay (msec)[b]	217 ± 8	*	248 ± 8
Amplitude (%)[c]	4.9 ± 0.4	***	0.4 ± 0.4

[a] Data reported as mean ± SEM.
[b] The delay between stimuli of a paired pulse presentation that elicits the largest amplitude aftercontraction.
[c] Percentage of control, control being the amplitude of the response to the first stimulus of the pair at the optimal delay.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

et al. (16) who report that the hypertrophy that accompanies the hypertension in SHRs up to 24 weeks of age is associated with the "stable peak pumping ability" of the heart when expressed per gram of myocardium.

While isometric characteristics of single contractions do not differ between SHRs and WKYs, the way in which two mechanical responses interact is different. The prolonged mechanical refractory period (MRP) seen in the SHRs reflects an altered time course of recovery events associated with a twitch. One component of this interval is the electrical refractory period (ERP) of the membrane. The longer MRP of SHRs might reflect a prolonged ERP. This possibility is supported by a report of a prolonged action potential plateau phase in ventricular cells from SHR preparations (12).

The MRP may also depend upon the time course of replenishment of the "activator Ca^{++} stores" of the sarcoplasmic reticulum. This is the major source of Ca^{++} for coupling excitation to contraction although a significant amount of extracellular Ca^{++} does move into the cell during the action potential (18). If the net rate of filling of this store is depressed, the MRP may be prolonged. Recent studies (19) indicate that Ca^{++}-uptake and -binding by sarcoplasmic reticulum from SHR cardiac muscle is de-

pressed. Yet, with paired pulses in this study, as the delay was increased, no differences were detected between SHR and WKY muscles. Since the amplitudes of mechanical events observed in this period are most likely to reflect the amount of Ca^{++} available for contraction, the results of this aspect of the study alone do not point to obvious differences in the rate of Ca^{++} recycling between SHR and WKY muscle.

An intriguing finding of this study was the accentuated frequencies of occurrence and strength of aftercontractions in the SHR preparations. The mechanism(s) responsible for aftercontractions is presently obscure. These mechanical events have been described by others to be exaggerated by high [Ca^{++}], epinephrine, and low temperature, yet are independent of propagated membrane voltage changes (20, 21). The observation that SHRs are more prone to develop aftercontractions leads to the suspicion that events associated with Ca^{++} cycling are altered in SHR heart muscle.

Summary. Isometric properties of the isolated cardiac muscle of spontaneously hypertensive rats (SHR) were compared to those from age-matched normotensive Wistar-Kyoto (WKY) preparations to assess the contribution of alterations in intrinsic contractile properties of cardiac muscle to the well-documented increase in cardiac output seen during the early phases of hypertension.

At L_{max}, there were no detectible differences in resting tension, active tension, rate of tension development, or rate of relaxation between SHR and WKY preparations, indicating that neither the hypertension nor the accompanying cardiac hypertrophy influences these particular contractile characteristics. However, interactions between individual contractions of cardiac muscle were different. With paired pulse stimulation, SHR preparations had longer mechanical refractory periods and larger and more frequent aftercontractions. Therefore, during the SHR's high cardiac output phase, certain, but not all, of the intrinsic mechanical

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)F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 483-487 (1977)

tic Catalase Is Not Essential for the Hypolipidemic Action of Peroxisome Proliferators[1] (39699)

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us studies demonstrated that a of structurally unrelated com- hat lower serum lipid levels in man experimental animals (2-4) cause a ncrease in hepatic peroxisome (mi- profiles in rats and mice (5-10). ease in peroxisomes was accompa- a significant elevation of hepatic and carnitine acetyltransferase ac-), 11, 12). The frequent association hepatic peroxisome proliferation olipidemia strongly suggested the y that either peroxisome catalase other peroxisomal enzyme may be in lipid metabolism (8, 9).

resent studies were conducted to the role of hepatic peroxisomal cat- he hypolipidemic effect exerted by xisomal enzyme inducers. These ids were administered either in tion with allylisopropylacetamide which inhibits the catalase synthesis with aminotriazole (AT), which s irreversibly with catalase and in- this enzyme (14). We now report bstantial reduction in serum lipids in rats, although the liver catalase vas markedly diminished, and that ferated peroxisomes lacked cyto- ly demonstrable catalase.

als and Methods. Inbred male F- (Simonson Laboratories, Inc., Gil- if.) weighing between 135-175 g ed in these studies. The animals ce-fed a semisynthetic diet as de- reviously (15). The hypolipidemic lofibrate (100 mg/kg body wt), lofenapate (10 mg/kg body wt), or nefenopin {2-methyl-2[*p*-(1, 2, 3, 4-tetrahydro-1-naphthyl)phenoxy]-propionic acid, Su-13,437} (50 mg/kg body wt) were administered by gavage twice daily at 12-hr intervals. Allylisopropylacetamide (AIA) (Hoffman-LaRoche, Inc., Nutley, N.J.), dissolved in saline, was given sc in a dose of 200 mg/kg body wt every 12 hr. 3-Amino-1,2,4-triazole (K & K Laboratories, Plainview, N.Y.) was dissolved in water and injected ip (1 g/kg body wt) twice daily. Each group consisted of five rats (Fig. 1).

The serum total cholesterol and glyceride glycerol were determined by the method of Azarnoff (16). Liver catalase activity was assayed by the spectrophotometric method of Lück (17) as previously described (7). The activity of carnitine acetyl transferase (CAT) was measured on high-speed supernatants of sonified rat liver homogenates, using the thio-acceptor DTNB [5,5'-dithiobis(2-nitrobenzoate)]. The release of CoA from acetyl CoA, in the presence of carnitine, was measured at 412 nm (12). Total protein was measured by the method of Lowry *et al.* (18).

Liver for electron microscopy was fixed in 2.5% glutaraldehyde, buffered with 0.1 *M* sodium cacodylate, pH 7.4, for 4 hr and rinsed overnight in the cacodylate buffer. The finely chopped sections were incubated in the 3,3'-diaminobenzidine medium of Novikoff and Goldfischer (19), postfixed in 2% OsO_4, and processed for electron microscopy (7).

Results. The effect of combined administration of AIA and the peroxisome proliferators on liver catalase and serum lipids. Figure 1 shows the results of AIA administration in combination with the peroxisome proliferator clofibrate, methyl clofenapate, or nafenopin for 7 days. The expected increases in liver weight and catalase activity were observed in rats treated with the hypo-

rted in part by United State Public Health ants GM-23750 and GM-15956.

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ISSN 0037-9

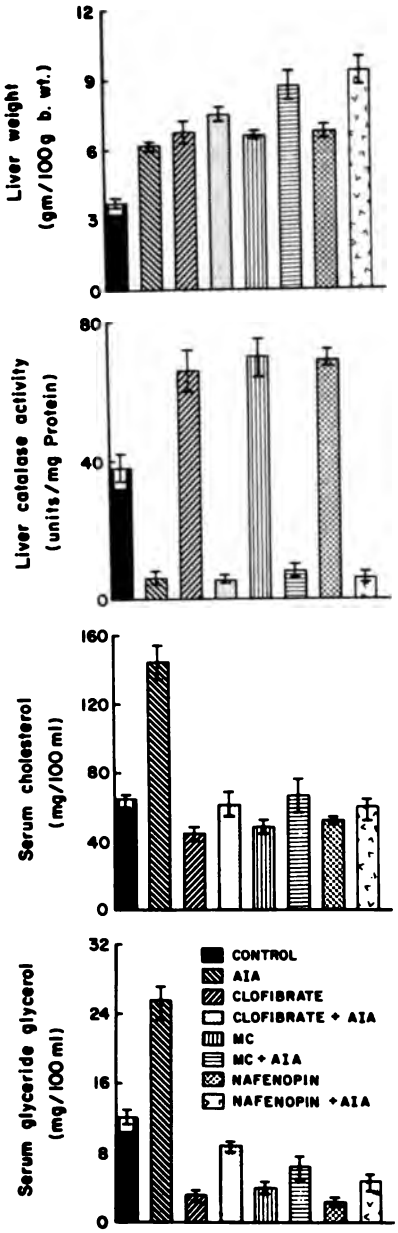

FIG. 1. Changes in liver weight, liver catalase activity, and serum lipids in rats treated with allylisopropylacetamide (AIA) and the hypolipidemic drugs clofibrate, methyl clofenapate (MC) and nafenopin. Rats received the hypolipidemic drug by gavage twice daily; AIA was injected sc also twice daily for 7 days. Doses are given in the text. Each bar represents the mean ± SE of five rats in each group.

lipidemic drugs clofibrate, methyl clofenapate, or nafenopin. In contrast, the liver *catalase activity* decreased markedly in animals given AIA alone or in combination with the peroxisome proliferators. A significant hypercholesterolemic as well as hypertriglyceridemic effect was observed in rats treated with AIA alone, confirming the results of other workers (20, 21). On the other hand, rats to which AIA was administered with clofibrate, methyl clofenapate, or nafenopin, had significantly lower serum lipids than those which received AIA alone ($p < 0.001$), although the liver catalase activity in these animals was almost completely abolished. A marked proliferation of hepatic peroxisomes, however, occurred in these animals given combined treatment, but these organelles did not contain cytochemically demonstrable catalase (Fig. 2B). As expected, the proliferated peroxisomes in animals treated with only clofibrate, methyl clofenapate, or nafenopin showed intense reaction product, when incubated in the 3,3′-diaminobenzidine medium (Fig. 2A). The peroxisomes in the livers of rats treated with AIA alone for 7 days were smaller than normal and showed no increase in their numbers (15, 22).

Hepatic carnitine acetyltransferase activity (CAT) in rats treated with clofibrate and allylisopropylacetamide (AIA) or with aminotriazole (AT). CAT activity was assayed following the administration of clofibrate with AIA or with AT to ascertain if this enzyme is increased in proliferated peroxisomes which lacked catalase. The CAT activity increased significantly in rats treated with clofibrate alone or in combination with AIA or AT (Table 1). The administration of AIA or AT alone for 7 days did not increase the activity of this enzyme.

Discussion. In an earlier study (15) we have shown that when clofibrate was administered to rats receiving AIA, an agent that blocks the synthesis of catalase (13), the proliferation of peroxisomes occurred even though the liver catalase activity was negligible. The present studies extend this observation with other peroxisome proliferators and demonstrate that peroxisomes proliferated under the influence of AIA lacked cytochemically demonstrable catalase. It is to be noted that the cytochemical staining reaction in peroxisomes is the result of peroxidatic oxidation of 3,3′-diaminobenzidine by the enzyme catalase. The absence of cyto-

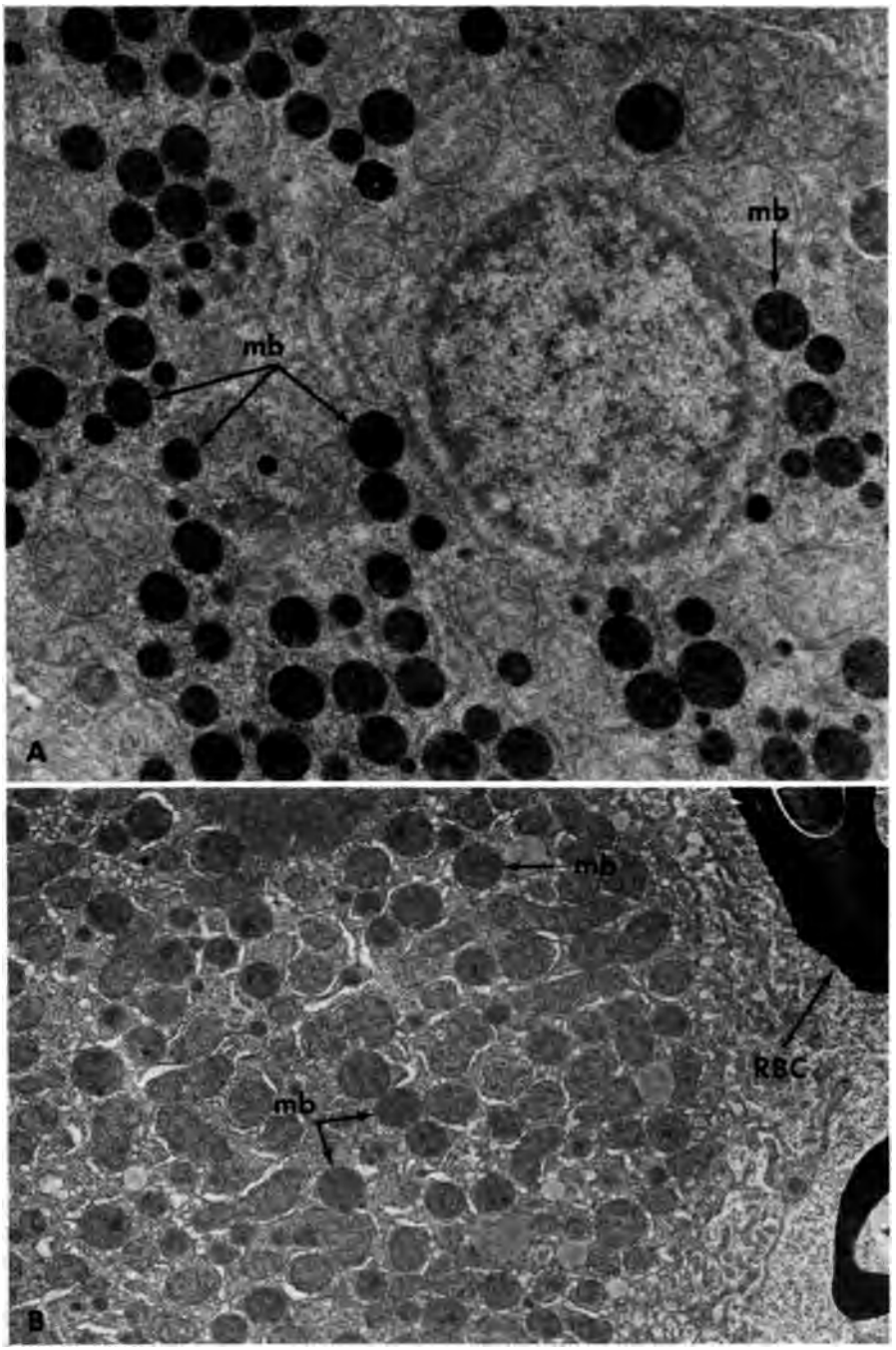

FIG. 2. A. Rat treated with clofibrate for 7 days. The hepatic peroxisomes (microbodies, mb) are increased in number and display intense reaction product after incubation for 30 min at 37° in the alkaline 3,3'-diaminobenzidine medium, pH 9.0. × 14,800. B. Rat treated with AIA and clofibrate for 7 days. Peroxisome (mb) proliferation is evident, but these organelles contain very little reaction product after incubation in the diaminobenzidine medium as in Fig. 2A. The red blood cells (RBC) in the sinusoids stained positively because of the peroxidatic activity of hemoglobin. × 12,500.

TABLE I. EFFECT OF ADMINISTRATION OF CLOFIBRATE WITH ALLYLISOPROPYLACETAMIDE (AIA) OR WITH AMINOTRIAZOLE (AT) ON HEPATIC CARNITINE ACETYLTRANSFERASE ACTIVITY IN F-344 Male Rats.

Group[a]	Number of animals	Carnitine acetyltransferase activity (units/mg of protein)[b]
Control	4	3.3 ± 0.4
Clofibrate	4	128.9 ± 12.6*
AIA	5	4.2 ± 1.4
Clofibrate + AIA	4	43.1 ± 3.3*
AT	5	5.9 ± 1.7
Clofibrate + AT	5	80.9 ± 6.5*

[a] The drugs were administered for 7 days: clofibrate, 100 mg/kg body wt, twice daily by gavage; AIA, 200 mg/kg body wt twice daily by sc injection; and AT, 1000 mg/kg body wt twice daily by ip injection.
[b] Mean ± SE.
* Significantly different from controls or from AIA- or AT-treated animals ($p < 0.001$).

chemical staining reaction in peroxisomes as well as the marked reduction in catalatic activity appear to be the result of low levels of catalase protein in the livers of rats treated with AIA and the hypolipidemic peroxisome proliferators. By immunoprecipitation method, the amount of catalase protein in these animals was found to be markedly diminished (unpublished observation). It is also clear from these studies that there is a significant serum lipid lowering effect in rats treated concurrently with a hypolipidemic agent and AIA, despite the abolition of catalase in proliferated hepatic peroxisomes and the very low activity of this enzyme in liver homogenates.

The studies of Caravaca *et al.* (23) demonstrated that hepatocatalase peroxidase, an active peroxidase-oxidase subunit isolated from beef liver catalase, induced a hypocholesterolemic effect in rabbits. This observation, prompted Svoboda and Azarnoff (6) to suggest that the hypolipidemic effect of clofibrate might be mediated by the endogenous increase of liver catalase resulting from peroxisome proflieration. Furthermore, the lower levels of serum lipids in a strain of acatalasemic mice were attributed to unstable catalase that displayed increased *in vivo* peroxidatic activity (24). In the present studies a substantial serum cholesterol and triglyceride lowering effect was evident in animals treated simultaneously with AIA and peroxisome proliferators, suggesting that peroxisome catalase is not necessary for the mediation of hypolipidemic action. A recent study on the effect of 3-amino-1,2,4-triazole on lipid metabolism also indicates that catalase itself is not directly related to lipid metabolism (25).

The observation that peroxisome catalase is not essential for the hypolipidemic action of these drugs should not be construed as evidence that peroxisome proliferation is not related to lipid metabolism. Because peroxisome proliferation can be induced in the absence of catalase synthesis, Reddy *et al.* (15) suggested that this might be due to the possible induction of yet unidentified proteins that make up between one-half and two-thirds of the total peroxisome proteins (26). The increase in hepatic CAT activity in rats treated simultaneously with AIA and clofibrate, in which the peroxisomes were deficient in catalase, is an indication that other peroxisomal enzymes are induced by these hypolipidemic drugs. The CAT has been identified in rat liver peroxisomes (27), and we have shown that all agents capable of inducing peroxisome proliferation also increase the activity of this enzyme (12). Lazarow and deDuve (28) have recently detected the fatty acyl-CoA oxidizing system in rat liver peroxisomes and showed that this activity increased in rats treated with clofibrate. It appears that synthesis of these and possibly other peroxisomal enzymes, but not catalase, may be responsible for the hypolipidemic effect of these peroxisome proliferators.

Summary. To evaluate the role of peroxisomal catalase in lipid metabolism the hypolipidemic drugs clofibrate, methyl clofenapate, and nafenopin were administered to rats in combination with allylisopropylacetamide, an agent which inhibits catalase synthesis. The number of peroxisomes in liver cells was increased, but these organelles did not contain cytochemically demonstrable catalase. A marked reduction in liver catalase activity was also noted. Although the proliferated peroxisomes were deficient in catalase activity, a substantial hypolipidemic effect was observed in these animals, suggesting that peroxisome catalase is not nec-

essary for the hypolipidemic effect. The increase in carnitine acetyltransferase activity in these animals emphasizes the need to delineate other peroxisomal enzymes which may be involved in lipid metabolism.

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Received October 18, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 488-492 (1977)

Studies on the Intestinal Absorption of Bovine Xanthine Oxidase[1] (39700)

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Xanthine oxidase is a two-subunit (mol wt 150,000/subunit) enzyme that catalyzes the conversions of hypoxanthine to xanthine and xanthine to uric acid. It occurs in several organs of the body, especially the liver and small intestine (1). Xanthine oxidase is found in high concentrations in bovine milk, and it has been suggested that this enzyme from homogenized milk is absorbed intact from the small intestine and is instrumental in the development of atherosclerosis (2, 3). Evidence presented has included a correlation, in some of the countries considered, between homogenized milk consumption per capita and the incidence of coronary artery disease (4). Also, xanthine oxidase activity has been found in human atherosclerotic tissues obtained at autopsy (5). It is purported that homogenization of milk reduces the size of the fat particles, which contain the xanthine oxidase, to such a degree that they are readily absorbed without being hydrolyzed (2, 4). Many objections to this hypothesis exist (6); the purpose of this work is to investigate the possibility that xanthine oxidase may be carried through the stomach and absorbed intact by the small intestine.

If xanthine oxidase is to be absorbed by the small intestine, it must first pass through the stomach. This would require that the enzyme resist hydrolysis when held at the pH of the stomach for a time comparable to its transit time. If the xanthine oxidase gets through the stomach, it faces a barrier to absorption in the small intestine because of its large size; macromolecules are not routinely absorbed (7). There is, however, some evidence for absorption of intact pancreatic enzymes (mol wt 25,000) (8, 9).

We have investigated the fate of xanthine oxidase at the pH of the stomach using a low pH buffer, and we have studied the absorption of the enzyme by the everted rat intestine.

Materials and methods. Xanthine oxidase was assayed as follows (10, 11). An aliquot of 100 μl of the solution or suspension to be assayed was combined with 100 μl of 0.5 *M* Trizma buffer (pH at 37°, 8.31; Sigma Chemical Co., St. Louis, Mo.) and 100 μl of [8-^{14}C]xanthine solution (2.22×10^8 dpm/ml; ICN Pharmaceutical, Irvine, Calif.). The radioactive xanthine was diluted with 0.025 *M* xanthine to different specific activities in the different experiments: It was diluted to 3.6×10^4 dpm/μl in the acid denaturation experiment, to 4.5×10^4 dpm/μl in the everted gut experiment, and to 3.3×10^4 dpm/μl in the homogenized gut experiment. The following volumes of milk were assayed for xanthine oxidase activity: 0, 15, 45, and 150 μl. The first three volumes were made up to 100 μl with Krebs buffer (millimolarities: NaCl, 128; KCl, 5.1; $CaCl_2$, 2.3; KH_2PO_4, 1.3; $MgSO_4$, 1.4; $NaHCO_3$, 25.6; D-glucose, 9.0), and then combined with 100 μl of Trizma buffer and 100 μl of [^{14}C]xanthine solution for the assay. The 150 μl of milk was made up to 200 μl with Trizma and combined with 100 μl of [^{14}C]xanthine solution.

These solutions were shaken at 37° for 30 min in a Dubnoff metabolic shaking incubator. The incubation was stopped by the addition of 50 μl of 2.3 *M* $HClO_4$. Samples were centrifuged at 1500 rpm for 20 min. A 140-μl aliquot was taken and added to 20 μl of 1:1 solution of 0.025 *M* xanthine and 0.05 *M* uric acid. Separation of xanthine from uric acid was accomplished by spotting 10 μl of this solution, in five increments, on a cellulose thin-layer chromatogram (No.

[1] Supported in part by General Research Support Grant 5-SO7-RR-05456.

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ISSN 0037-9727

Eastman Kodak, Rochester, N.Y.). were prepared by adding 10 μl of spension to 15 ml of Aquasol liquid tion cocktail (New England Nuclear, Mass.) and counting as described The chromatogram was developed 5 hr in 2:1:1 *n*-BuOH, CH_3COOH, O, and allowed to dry overnight. e and uric acid spots were visualized ltraviolet light and scraped into cenubes. Scrapings were shaken with 5 0% NH_4OH, centrifuged at 1500 2–3 min and the supernatant was d into liquid scintillation vials. A three extractions of each sample was ed. This procedure routinely re-90% of the radioactivity from the ogram. The NH_4OH solutions were ted at 70° in a Brinkmann (West-.Y.) sample concentrator to a volless than 1 ml. Aquasol (15 ml) was nd the samples were counted three or 5 min in a Packard (Downers Ill.) Model 3330 liquid scintillation neter. Disintegration rates (dpm) lculated by correctly for the effiof the instrument (86%) and for through the use of an external stan-

ine oxidase activity is expressed as les of uric acid formed per hour and ers of incubation medium. The per- of spotted activity recovered from hine and uric acid spots was 80% or Ten percent of that not recovered in the extraction process. It is likely remainder was elsewhere on the ogram. Other regions of the chrm were not routinely assayed.

rsity of Wisconsin homogenized vi- milk and pH 1.75 buffer (0.108 *M* 0.050 *M* in KCl) were used in the naturation experiment. Eight milkuspensions were held at 37° for 15 ur of these were composed of 1 ml and 9 ml of buffer and the remaining re composed of 5 ml of milk and 5 uffer. Aliquots of 1.5 ml from each ion were neutralized with either 1.5 of Trizma buffer (pH 8.31).

H of all eight milk-buffer suspene eight aliquots neutralized with 1.5 izma, and the eight aliquots neutralized with 3 ml of Trizma was measured with a Corning (Corning, N.Y.) Model 10 pH meter. Xanthine oxidase activity of all neutralized samples was determined as described above.

For the everted intestine experiment, four adult male Sprague-Dawley rats (Holtzman Farms, Madison, Wis.) were starved for 18 hr, then killed by cervical dislocation. The small intestine was removed and flushed with five 10-ml portions of cold Krebs buffer and everted over a glass rod. The intestine was cut into three 10-cm strips, which were filled with Krebs and tied at both ends. Two of the strips were incubated in 10 ml of Krebs and the third was incubated in 5 ml of Krebs plus 5 ml of milk. The assignment of oral, middle, and caudal strips to the incubation flasks was randomized among the different animals. The strips were incubated at 37° for 1 hr with 95:5 O_2–CO_2. After incubation, the strips were cut and the serosal fluid was withdrawn. The fluid from one of the two strips incubated in Krebs alone was combined with 50 μl of milk. All samples were dialyzed for 2 hr against 0.15 *M* KCl and assayed for xanthine oxidase activity. Also included in the xanthine oxidase assay were controls consisting of 100 μl of nonincubated Krebs.

Homogenized gut experiments followed the same procedure as everted gut experiments through the incubation of the intestinal strips, except that the strips were 15 cm long and six rats were used. After incubation the strips were weighed and homogenized in nine times their weight of Krebs, first in a chilled Waring Blendor for 1 min, then with a Potter-Elvehjem Teflon and glass homogenizer held in ice. A 50-μl aliquot of the homogenate of one of the two strips incubated in Krebs alone was combined with 50 μl of milk. Xanthine oxidase activity was determined.

Statistical analysis was done utilizing an analysis of variance and the Student-Newman-Keuls test to determine differences between the means (12). The significance level selected was $p < 0.05$.

Results. The xanthine oxidase activity of milk was determined by using three different volumes of milk and assaying for conversion of xanthine to uric acid. These results

showed that the enzyme activity was proportional to the volume of milk up to approximately 45 μl of milk. The amount of uric acid formed per hr per μl of milk was 7.75 nmole.

For the acid denaturation experiment, the xanthine oxidase activity for the milk-buffer suspensions is shown in Table I. For both mixtures and both Trizma volumes, the conversion was less than 1.5 nmole of uric acid/hr/μl of milk. The last column of the table indicates the expected xanthine oxidase activity if the enzyme activity of the milk present in the assay were fully expressed. The latter values were obtained from the aforementioned assay of xanthine oxidase activity of milk. None of the samples showed as much activity as expected if the enzyme were intact.

The results of the everted gut experiment are shown in Table II. There was no significant difference between those strips incubated in Krebs alone and those incubated in Krebs plus milk, nor was there a difference between either of these and the control. There was a difference between those samples with added milk and the control, indicating that the milk used did contain xanthine oxidase.

The purpose of the homogenized gut experiment was to investigate the possibility that xanthine oxidase is absorbed by the intestinal mucosa but does not traverse the intestinal wall and appear in the serosal medium. Results are shown in Table II. Note that any activity may have been due not only to the presence of the enzyme in the wall, but also to its presence in the serosal fluid. This accounts for the possibility that the enzyme may be present in both locations in amounts too small to be detected, but when enzyme activity from both locations is combined, it may be measurable. Again there was no significant difference between those strips incubated in Krebs and those incubated with milk. The differences of these values from the controls reflect endogenous xanthine oxidase activity in the intestinal wall.

Discussion. The results presented in Ta-

TABLE I. XANTHINE OXIDASE ACTIVITY OF MILK AFTER TREATMENT WITH ACID.[a]

Buffer/milk ratio	pH of buffer/milk suspension	Volumes of Trizma	Assayed suspension pH	Xanthine oxidase activity (nmole of UA/hr/μl of milk)	Expected xanthine oxidase activity (nmole of UA/hr/μl of milk)
9:1	1.78 ± 0.11	1	2.23 ± 0.01	0.75 ± 0.05	8.1
		2	3.18 ± 0.14	1.30 ± 0.23	8.1
1:1	2.71 ± 0.02	1	6.05 ± 0.16	0.11 ± 0.0	7.1
		2	7.56 ± 0.02	0.26 ± 0.04	7.1

[a] Buffer/milk suspensions were held at low pH, neutralized with Trizma, and assayed for xanthine oxidase activity at the pH indicated in column four. Values are means ± SE for four determinations. UA, uric acid.

TABLE II. XANTHINE OXIDASE ACTIVITY OF EVERTED RAT INTESTINE SEROSAL FLUID AND OF HOMOGENIZED EVERTED RAT INTESTINE.[a]

Incubation medium	Krebs	Krebs plus milk	Krebs; milk added	Control (no incubation)
Xanthine oxidase activity nmole of UA/hr/μl of serosal fluid	0.25 ± 0.05†	0.16 ± 0.04†	0.90 ± 0.05*	0.08 ± 0.01
Xanthine oxidase activity nmole of UA/hr/μl of milk-homogenate	1.08 ± 0.12*,†	0.95 ± 0.05*,†	4.87 ± 0.83*	0.09

[a] Values for serosal fluid were obtained as follows: Everted rat intestinal strips were incubated in Krebs buffer with or without milk. After incubation, milk (50 μl) was added to the serosal fluid of one of the strips incubated in Krebs. Serosal fluid of all strips was assayed for the xanthine oxidase activity. Values are means ± SE for four rats.

Values for milk-homogenate mixtures were obtained as follows: Everted rat intestinal strips were incubated in Krebs buffer with or without milk. Strips and contents were homogenized 1:10; milk (50 μl) was added to 50 μl of the homogenate of one of the strips incubated in Krebs alone. All homogenates were assayed for xanthine oxidase activity. Values are mean ± SE for six rats. UA, uric acid.

* Significantly different from appropriate control at $p < 0.05$.

† Significantly different from Krebs; milk added at $p < 0.05$.

ow that xanthine oxidase is inacti-
ɔon treatment with acid for 15 min
of less than 3. Two recent abstracts
ported that some xanthine oxidase
persists after *in vitro* incubation with
gastric juice (13) and aspirated hu-
ɜtric juice (14). In both cases the
of milk used was sufficient to raise
above 3.5. Ho *et al.* (14) state that
e inactivation of xanthine oxidase
ɔelow pH 3.35. These reports are
nt with the data presented herein
icate that xanthine oxidase activity
ɔersist in the stomach only after
ɔlumes of milk are consumed.

II shows that there is no difference
ıine oxidase activity of serosal fluid
ɛrted intestinal strips incubated with
ıout milk, and that there is no differ-
intestinal homogenates incubated
dentical conditions. These results
that the enzyme does not enter the
ıl wall in active form within the lim-
:tection of the assay.

ıinimum amount of milk detectable
: 0.160 nmole of uric acid/hr/μl of
luid (Table II, Krebs + milk) corre-
to 1.0 μl of milk in the 100 μl of
fluid assayed. This value was ob-
rom the slope of a plot of xanthine
activity in milk vs the volume of

ɔus objections to the xanthine oxi-
:ory of atherosclerosis have not been
n experimental evidence. Presented
:vidence to substantiate two of these
ɪns: (i) that xanthine oxidase does
in its enzymatic activity at the pH of
ıach in the presence of homogenized
ıd (ii) that the large xanthine oxidase
e is not detectably absorbed by the
testine of the rat.

ıe H^+ present in high concentration
tomach is known to protonate sev-
ıino acid groups and catalyze the
e action of pepsins (7). These two
result in conformational and cova-
ıanges in proteins. It is likely that
ɛ oxidase is no exception, since
xposed to low pH and returned to
ty, the enzyme from milk showed no
: activity.

he intestinal epithelium constitutes a barrier to absorption for large molecules; macromolecules that are not digested are not absorbed, e.g., cellulose (7). If xanthine oxidase were to pass intact through the stomach, it probably would also pass intact through the intestine.

One further point not considered here is that xanthine oxidase would be subject to the action of pancreatic enzymes in the intestine. These enzymes split certain peptide bonds in proteins and polypeptides. Xanthine oxidase, whether inert to gastric digestion or not, would have to encounter these enzymes as well.

Although the evidence presented here indicates that xanthine oxidase is not absorbed from the small intestine, the possibility remains that it is absorbed in active form in amounts too small to be detected by this assay.

Summary. Certain aspects of the xanthine oxidase hypothesis of atherosclerosis were studied. Xanthine oxidase activity was determined by liquid scintillation counting of [^{14}C]uric acid formed by the action of the enzyme on [^{14}C]xanthine. Results show that the proposal that xanthine oxidase passes through the stomach and is absorbed intact by the small intestine is implausible. The treatment of milk with a low pH buffer resulted in irreversible loss of xanthine oxidase activity. The incubation of everted rat intestine in buffer and in buffer–milk mixtures showed no difference in enzyme activity in serosal fluid or in intestinal homogenates.

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Received June 11, 1976. P.S.E.B.M. 1977. V

F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 493-495 (1977)

ıltered Response to Cyclic AMP Stimulating Hormones in Intact Human Leukemic Lymphocytes (39701)[1]

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ıction. Adenylate cyclase, in par- ɔreparations from lymphocytes of with chronic lymphatic leukemia ıas previously been shown by us to lowered basal activity and a dimin- ;ponse to prostaglandin (PG) E_1, (1). Reports of cyclic AMP and :ipation in cell division and matura- ·) and modulation of certain func- ymphocytes (5) suggested that the reduction in adenylate cyclase ac- l response to stimulation may prove t in the abnormal behavior of leu- nphocytes. In this report, we dem- observations with intact human /tes, normal and malignant, which 'urther that the response of adenyl- ıse to extracellular effectors has red in leukemic lymphocytes.

ls and materials. Isolation of lym- Normal human lymphocytes were from the plasma layer of freshly ɛparinized venous blood (1). The ıs allowed to settle in 15-cc screw- onical tubes at 37°. The lympho- e isolated by careful removal of the ısma fraction, which contained high ıations of lymphocytes. Only cell containing a minimum of 95% /tes were accepted for study. Leu- :lls were obtained similarly from y untreated patients with leuke- : lymphocytes were freed of plate- :peated washing and centrifugation

:ocyte incubation. The intact cells or neoplastic lymphocytes) were ated for 30 min at 37° in medium ontaining 20% calf serum. Unless : stated, 6×10^6 cells were used bation. Incubation was initiated with the addition to each tube of 0.05 ml of a solution containing the given stimulant or equivalent solution not containing the stimulant. Prostaglandins were placed into aqueous solution with the aid of $NaHCO_3$. The incubation was terminated with 70% $HClO_4$ (containing [^{3}H]cAMP, 0.1 pmole). The final concentration of the perchlorate was 3.5%. The samples were centrifuged and the supernatant containing cAMP was neutralized with 3 *N* KOH. The insoluble salt precipitate was removed by centrifugation and the supernatant was passed through Dowex 50 W-X2 (H^+), lyophilized, resuspended, and assayed with a radioimmunoassay (1). The cAMP content of the medium was minimal, 0.5 cc containing approximately 0.5 pmole. No difference in medium cAMP content was observed before or after incubation. The prostaglandins were kindly supplied by Dr. Pike of the Upjohn Co.

Results. Under the incubation conditions described, the CLL cells contained about twice the cAMP per cell of normal lymphocytes (Table I). The cAMP content of the leukemic cells showed a much wider range of sample variability.

Table I demonstrates the cAMP levels of normal and CLL lymphocytes in response to treatment with isoproterenol, epinephrine, and glucagon. The relative response (stimulated/basal) was also determined. Both of the catecholamines showed stimulation relative to the basal solution in normal cells; glucagon had no appreciable effect. The relative response to epinephrine was significantly reduced in the malignant cells. Glucagon, again, had no appreciable effect on cAMP levels. This is best demonstrated in the stimulated/basal ratio, which illustrates that the CLL response to epinephrine was reduced by a factor of at least 2. The response to isoproterenol also appears reduced. However, with isoproterenol, enough variations existed from sample to

ork was supported in part by NIH Grant

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77 by the Society for Experimental Biology and Medicine
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ISSN 0037-97

TABLE I. cAMP LEVELS IN NORMAL AND LEUKEMIC LYMPHOCYTES IN RESPONSE TO VARIOUS AGONI

Additions	Normal lymphocytes		Leukemic lymphocytes	
	(pmole of cAMP/10^7 cells) Mean	Stimulated/ basal	(pmol cAMP/10^7 cells) Mean	Stimulated/ basal
None	15.7 ± 1.3		28.5 ± 5.5	
Isoproterenol (5×10^{-5} *M*)	26.3 ± 4.3	1.7	28.2 ± 16.8	N.S.
Epinephrine (2×10^{-5} *M*)	34.8 ± 11.7	2.2	31.2 ± 4.8	1.1
Glucagon (1.4×10^{-7} *M*)	17.6 ± 1.4	1.1	27.2 ± 7.8	<1
Prostaglandin				
E_1 (5×10^{-5} *M*)	121.0 ± 39.0	7.7	54.0 ± 13.0	1.9
E_2 (5×10^{-5} *M*)	127.0 ± 41.0	8.1	44.4 ± 20.6	1.6
$F_{2\alpha}$ (5×10^{-5} *M*)	85.2 ± 44.8	5.1	42.0 ± 10.0	1.5

[a] After a 30-min preincubation at 37°, 6×10^6 lymphocytes in 0.45 ml of medium "199" containing serum were exposed to 0.05 ml of a solution containing the given hormone. The cultures were then in fixed with PCA, and assayed for cAMP as described in the text. The hormone concentrations indicate final concentrations. These concentrations were determined to produce maximal stimulation in both th and leukemic cells. Equivalent solutions not containing the given hormone were added to the control cult addition had no effect on the cAMP content of these untreated cultures. N.S. not significant. Values rep leukemic and 12 normal samples. Each sample was determined in triplicate. *P* Values were calculated fo basal vs leukemic basal, and normal (stimulated/basal) vs leukemic (stimulated/basal) for each hormon

sample to make this observation statistically questionable.

In the case of prostaglandins, the altered response in the CLL leukocytes is dramatic. In normal lymphocytes, prostaglandins E_1 and E_2 showed approximately an 8-fold stimulation over basal. Prostaglandin $F_{2\alpha}$ was less active, and showed about a 5-fold stimulation over basal. The leukemic cells displayed a decreased response to all three prostaglandins tested to about 1/3 to 1/4 of that found in normal lymphocytes.

The prostaglandin stimulation of lymphocytes was not β-adrenergic. Propranolol (5×10^{-6} *M*), a β-adrenergic blocker, inhibited isoproterenol and epinephrine stimulation by 80 and 95%, respectively, but did not affect cAMP levels consequent to PG stimulation. These results indicate that the adenylate cyclase response of the CLL lymphocytes to effectors is very poor.

Discussion. The breakage of intact cells to produce particulate preparations often causes artifactual results in basal and signal-responding activity of adenylate cyclase. For this reason, to substantiate our previous results obtained with particulate preparations, we proceeded to determine the effect of prostaglandins and other extracellular effectors on cellular cAMP levels in intact, viable lymphocytes from normal and leuker jects. Our present results again sho duced stimulation of cAMP levels kemic cells by PGE and $PGF_{2\alpha}$. Th sults may prove meaningful, in view reported regulatory roles of the P(cyclic nucleotides in the immune re and cell proliferation of lymphocytes

Since the reduction in the cAMP re in CLL cells is not limited to the P also occurs with other effectors, our further suggest that a more generali fraction to extracellular signals may curring in the CLL lymphocytes. M the function of the lymphocyte oc response to extracellular proding (8)

The increased basal cAMP levels we found in intact leukemic cells, posed to the decreased basal adenyl clase content in particulate prepa from leukemic cells, which we report merly, represent a contradiction wh cannot explain with the available dat possible that the latter was a result of pronounced destruction of adenylate in leukemic cells during cell breakag other point to be considered with the is that human chronic lymphatic le has been described as a B-cell dise 10). More recently, this conclusion ha

modified somewhat to include a possible T-cell etiology, in some CLL cases (11). The normal lymphocyte population studied in this report contained a mixture of B and T cells. Data comparing the cAMP systems of B and T human lymphocytes and their response to extracellular effectors will have to be obtained in future experiments.

Summary. Lymphocytes from normal subjects and patients with chronic lymphatic leukemia were assayed for cyclic AMP content and their response to effector stimulation, in terms of increased cellular cyclic AMP levels. Concentrations of isoproterenol, epinephrine, and prostaglandins E_1, E_2, and $F_{2\alpha}$ which produced potent responses in normal lymphocytes were much less effective in increasing cyclic AMP levels of leukemic lymphocytes. The response to PGE_2 was depressed from an 8-fold stimulation over basal, in the normal lymphocyte, to a 1.6-fold response in the leukemic lymphocyte. These data, along with those obtained previously with particulate preparations, indicate that a dysfunction in receptor–adenylate cyclase interaction may exist within the membrane of leukemic cells.

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Received December 12, 1975. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 496-499 (1977)

The Absence of Tryptophan Hydroxylase Activity in Blood Platelets (39702)

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Introduction. Blood platelets accumulate, store, and can release the biogenic amine neurotransmitters serotonin, norepinephrine, and dopamine (1-5). These properties have led to a consideration of the use of the blood platelet as a model of a neuron (1-5). Platelets have an avid uptake mechanism for serotonin (6-9) and it is thought that platelet serotonin is largely derived from serotonin entering the plasma from the intestinal mucosa and other tissues (10).

It has also been reported (11) that platelets contain tryptophan hydroxylase, one of the enzymes required for serotonin synthesis from tryptophan. It therefore appeared that in addition to active uptake and storage of serotonin, platelets may also have the capacity to synthesize this amine from tryptophan.

Since the synthesis of serotonin by platelets is an important point in the understanding of these organelles, we decided to investigate this problem with the current more sensitive and specific assay systems for tryptophan hydroxylation.

Methods. Platelet-rich plasma (PRP) was prepared by centrifugation of heparinized whole blood obtained from human volunteers or male Sprague-Dawley rats at 200*g* for 15 min at 4°. The resulting PRP was again centrifuged at 200*g* for 15 min at 4° to remove any contaminating leukocytes. Examination of this final PRP by light microscopy (12) revealed little if any contamination by leukocytes. Platelets were sedimented at 2500*g* for 15 min at 4° and washed twice in 50 m*M* Tris-Cl, pH 7.4, 100 m*M* NaCl, 2 m*M* dithiothreitol by resuspension-centrifugation. The final platelet pellet was resuspended in a small volume of 50 m*M* Tris-Cl, pH 7.4, and 2 m*M* dithiothreitol, sonicated essentially as described by Wolf and Shulman (13), and centrifuged at 27,000*g* for 15 min at 4°. The resulting supernatant fractions were retained for assay. Rat pineal glands and brain regions were homogenized in 50 m*M* Tris-Cl, pH 7.4, and 2 m*M* dithiothreitol and similarly centrifuged.

Tryptophan hydroxylase activity was determined in tissue extracts by the method of Friedman *et al.* (14) as modified by Baumgarten *et al.* (15) with volume alterations to give a more sensitive microassay. A 0.05-ml portion of platelet extracts or 0.02 ml of pineal, tegmentum, or striatum extracts was incubated in a final volume of 0.08 ml containing 12 μmole of Tris-Cl, pH 7.4, 0.4 μmole of dithiothreitol, and 17 nmole of 6-methyltetrahydropterin (Calbiochem) at 37° for 5 min. The reactions were initiated by the addition of 0.02 ml of 2 m*M* tryptophan. After 40 min the reactions were stopped by the addition of 0.02 ml of 6 *N* perchloric acid and centrifuged to remove the precipitated protein. A 0.1-ml portion of supernatant was mixed with 0.03 ml of concentrated hydrochloric acid. The fluorescence emission of the solution was measured at 540 nm with an excitation wavelength of 290 nm and compared with 5-hydroxytryptophan standards. The activity of tryptophan hydroxylase was measured as the difference in activity with and without 6-methyltetrahydropterin cofactor in the reaction mixture.

The tryptophan hydroxlase activity of cultured rat pineal gland and human and rat platelet-rich plasma was determined essentially by the procedure of Hakanson and Hoffman (16). Briefly, rat pineals were cultured in 60 × 15-mm culture dishes containing 2.5 ml of BGJ_b-Fitton-Jackson medium (Grand Island Biological Co.) supple-

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ISSN 0037-9727

d with 100 μg/ml of ascorbic acid and glutamine. The pineal cultures and t-rich plasma were incubated with 4 l of L-tryptophan (side chain-3-^{14}C; Ci/mmole; New England Nuclear in the presence of either 10^{-4} *M* 1055 (3-hydroxybenzylhydrazine; Nephew Research, Ltd.), an aromatic carboxylase inhibitor, or 10^{-4} *M* par- (Abbott Laboratories), a monoamine e inhibitor, for 2 hr at 37° under an phere of 95% oxygen–5% carbon e. At the end of the incubation period telets were sedimented and washed in *1* Tris–Cl, pH 7.4, 100 m*M* NaCl, 2 thiothreitol at 2500*g* for 15 min at 4°. ineal glands were blotted free of me- The pineals and platelet pellets were ted with 2 ml of acetone–1 *N* HCl containing 50 μg each of nonradioac- yptophan, 5-hydroxytryptophan, and nin. The extracts were pipetted free atured pineal or platelet protein and rated under nitrogen. The dried resi- were dissolved in a minimal volume ximately 25 μl) of acetone–1 *N* HCl and applied to 2-mm-thick 20 × 20- lica gel thin-layer plates (Merck, stadt) and developed in the ascending on with 14% sodium chloride (16). lates were scraped in 5-mm sections cintillation vials and counted with 10 Instabray (Yorktown Research).

ults. The tryptophan hydroxylase ac- of platelet extracts were compared to tivities found in the rat pineal and ntal region of the brain (high trypto- hydroxylase activity) and the striatal of the brain (relatively low trypto- ydroxylase activity). As seen in Table apparent tryptophan hydroxylase ac- of both rat and human platelets is ximately 0.1–0.45% of the activity in the rat pineal and 0.17–0.75% of t tegmentum activity, and was not sig- ntly higher than the blank. The appar- zyme content is also considerably less he activity found in the striatum. The phan hydroxylase activities of plate- ported here are 1/50 to 1/100 of the ies reported previously (11).

ontrol experiments it was found that on of serotonin to tegmental extracts did not inhibit the hydroxylase activity. This eliminates the possibility that the high endogenous serotonin content of the platelet extract inhibits enzyme activity. Further, the mixing of platelet and tegmental extracts did not enhance or inhibit the tryptophan hydroxylase activity of the tegmental extract. This eliminates the possibility that an endogenous hydroxylase inhibitor is being liberated during the sonic disruption of the platelets. Finally, the sonication conditions for platelets did not affect the tryptophan hydroxylase activity of a similarly treated tegmental extract.

Since platelet extracts did not seem to have tryptophan hydroxylase activity it was decided to see if intact platelets in a platelet-rich plasma could convert labeled tryptophan to serotonin. The rat pineal is capable of converting tryptophan to 5-hydroxytryptophan in culture in the presence of a decarboxylase inhibitor (16; Fig. 1). Furthermore, the total pathway of tryptophan conversion to serotonin may be monitored in the cultured pineal in the presence of the monoamine oxidase inhibitor pargyline (see Fig. 1). This assay system was utilized with platelet-rich plasma. Results in Table II indicate that the cultured rat pineal is capable of hydroxylating tryptophan in the presence of the decarboxylase inhibitor NSD 1055 while platelets cannot. The tryptophan hydroxylase activity of rat and human platelets was found to be 0.35–0.57% of the rat pineal activity which is in reasonable agreement with the relative activities shown in Table I. The tryptophan hydroxylase activity of the cultured rat pineals was found to be similar to those reported by Hakanson and Hoffman (16). In the presence of the mono-

TABLE I. TRYPTOPHAN HYDROXYLASE ACTIVITY OF TISSUE HOMOGENATES.[a]

Source of tissue	Tryptophan hydroxylase activity[b]	
Rat pineal	26.8 ± 3[r]	*n* = 3
Rat tegmentum	16.2 ± 1.9	*n* = 4
Rat striatum	0.6 ± 0.12	*n* = 4
Rat platelets	0.03 ± 0.05	*n* = 6
Human platelets	0.12 ± 0.09	*n* = 4

[a] Tissue and platelet extracts were prepared and assayed for tryptophan hydroxylase as described.

[b] Activity is expressed as the nanomoles of 5-hydroxytryptophan formed per milligram of protein per hour.

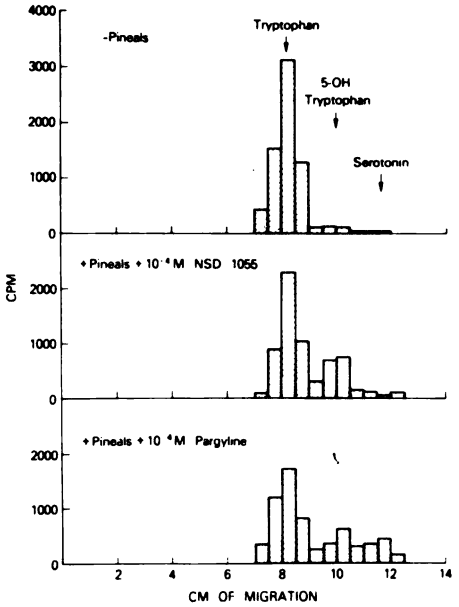

FIG. 1. The synthesis of 5-hydroxytryptophan and serotonin by cultured rat pineals. Pineals were cultured with [^{14}C]tryptophan in the presence of 10^{-4} *M* NSD 1055 or 10^{-4} *M* pargyline as described. Tryptophan metabolites were separated by thin layer chromatography on silica gel layers with 14% sodium chloride (16). The migration of tryptophan, 5-hydroxytryptophan, and serotonin is indicated.

amine oxidase inhibitor pargyline, there was no conversion of labeled tryptophan to 5-hydroxytryptophan or serotonin by platelet-rich plasma (data not shown).

Discussion. From the results presented above it appears that platelets do not have the capacity to synthesize significant amounts of serotonin *de novo* from tryptophan. The reason for the difference between these results and the previous ones from this laboratory (11), indicating the presence of tryptophan hydroxylating capability for platelet extracts, is unexplained. However, the earlier work was done with an assay which at that time was just being developed, and with relatively small amounts of platelet protein. Thus, while the specific activity previously reported (11) was fairly good, it represented only a small amount of apparent enzyme and was probably an artifact of the assay system.

The lack of serotonin synthetic capacity in platelets means that they do not represent a

TABLE II. TRYPTOPHAN HYDROXYLASE AC INTACT RAT PINEAL GLANDS AND RAT AND PLATELETS.[a]

Source of tissue	Tryptophan hydroxylas
Rat pineals	3.68 ± 0.4
Rat platelets	0.013 ± 0.01
Human platelets	0.021 ± 0.01

[a] Tryptophan hydroxylase activity was d in cultured rat pineal glands or in rat or huma rich plasmas in the presence of 10^{-4} *M* NSI described.

[b] Activity is expressed as the percentage tryptophan converted to 5-hydroxytryptopha ligram of tissue protein per hour.

complete model of the serotonin although the serotonin uptake and may resemble that found in nerve (5). For this latter purpose the plate still be considered an adequate mod

Summary. Under conditions that maximal tryptophan hydroxylating in extracts from pineal glands and t hydroxylase activity could be dete platelets. Furthermore, with syste are adequate to measure serotonin s in cultured pineal glands or whole serotonin synthetic capacity could tected in human or rat blood platel

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Received September 27, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 500-504 (1977)

Response of Left Ventricular Mechanoreceptors to Changes in Pressure and Muscle Length (39703)[1]

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Previous studies have provided evidence for afferent sympathetic nerves from the heart which traverse the left upper thoracic white rami communicantes. Anatomically, these fibers have been studied by Mizeres (1), and sensory endings of sympathetic nerve fibers have been histologically located by King (2) and Khabarova (3). Cardiac sympathetic afferent nerve activity has been reported to be spontaneous and can be stimulated by various chemical and mechanical means (4–6). Tonic activity of sympathetic afferents related to normal cardiac events and entering the spinal cord at T-3 has also been noted (6, 7). Recent studies in our laboratory by Hess *et al.* (8) have specifically related cardiac afferent sympathetic nerve activity to isolated changes in peak-developed left ventricular pressure.

The purpose of these experiments was (i) to measure left ventricular mechanoreceptor response to changes in left ventricular volume and pressure, and (ii) to correlate sympathetic afferent nerve activity in left thoracic white rami communicantes with changes in left ventricular pressure and muscle length during the cardiac cycle. After cardiopulmonary bypass, cardiac afferent nerve discharge was studied during isovolumetrically developed changes in left ventricular pressure and during isobarically developed changes in myocardial muscle length.

Materials and Methods. Afferent nerve activity was studied in 14 small single and multifiber nerve preparations in 11 mongrel dogs (25–35 kg) anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories), 30 mg/kg intravenously. The animals were intubated and ventilated with 40% oxygen in air, using a Bird Mark VII respirator. The animals were placed on total cardiopulmonary bypass (Fig. 1). In the following, the procedures and methods, which have been previously described in detail (8), are outlined. A cannula in the right ventricle allowed drainage of coronary venous blood. The right and left lungs were tied at the hilum, and aortic perfusion pressure was maintained at approximately 90 mm Hg using a Sarns roller pump. During bypass, arterial blood gases and pH were maintained between 7.35–7.45 pH, 38–42 mm Hg PCO_2, and PO_2 greater than 200 mm Hg. Muscle relaxation was obtained with 20 mg of gallamine triethiodide (Flaxedil, Davis and Geck). This dose was repeated every 45 min along with supplementary doses of sodium heparin (3000 IU) and Nembutal as required.

The heart was supported with a pericardial cradle. Electrocardiographic electrodes were sewn directly into the myocardium using stainless steel monostrand suture.

Two balloons, one liquid filled for isovolumetric and one air filled for isobaric studies (designed to be used interchangeably), were pulled into the left ventricle through the ventricular apex and securely anchored to the apical endocardium. A drain for removal of Thebesian and bronchial venous blood was also inserted into the left ventricle. Balloon compliance was such that it did not significantly alter pressures within the expected range of measurements. Isobaric contractions of the left ventricle were studied using a closed system consisting of a balloon attached to wide-bore polyethylene tubing and connected to a source of air pressure and a 50-liter Nalgene bottle. A Fleisch

[1] This study was supported by the National Heart and Lung Institute (Grant HL16511), the Wisconsin Heart Association, and the Medical Research Service of the Veterans Administration.

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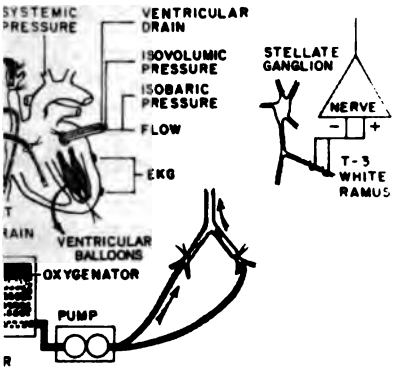
ISSN 0037-9727



Systemic perfusion system. SVC, superior IVC, inferior vena cava; R. Vent., right .V. drain, left ventricular drain; EKG, elec-ıphic electrodes.

ıchometer, a Statham PM97 dif-ɔressure transducer, and a Statham ressure transducer were placed in n for measurement of pressure and ıe isobaric apparatus permitted ın cardiac volume without associ-ŋges in intraventricular pressure. ar ejection into this closed system in system pressure fluctuations of 1%.

c flow curves and isovolumetric ıric ventricular pressures were re-ong with systemic arterial pressure rass polygraph. These parameters g systemic pressure) were recorded lberg Series 100 FM tape recorder. ta could then be replayed for com-lysis and also visually examined on vell Model 1912 visicorder. Arte-l gases and pH were analyzed on a ter Type PHA927b blood gas ma-

recording. The sympathetic chain ft upper thoracic region was ex-ıd a white rami communicantes, '-3, was isolated, stripped of con-ssue, and cut to eliminate efferent The peripheral end of the nerve ed into slips and placed on bipolar ; electrodes. Nerve activity was ırough a low noise AC preampli-ctive electronic variable band-pass d displayed on a Tektronix 564B ıpe. Nerve data was also stored on . Nerve amplifier output was used to drive a loudspeaker which facilitated recognition of cyclic nerve discharge.

Data acquisition. When nerve activity was found which cycled with cardiac rhythm: (i) Isobaric pressures were recorded with the isovolumetric balloon completely collapsed and pressures elevated in 25 mm Hg steps. Nerve activity was recorded and collected in steps for 32 nonarrhythmic heart beats up to the final recording pressure of 100 mm Hg. The isobaric balloon was then collapsed for isovolumetric studies using the same nerve fiber. (ii) Isovolumetric pressures were recorded in increments of 50 mm Hg peak systolic pressure. Nerve activity was recorded for 32 nonarrhythmic heart beats including a final recording at 150 mm Hg peak pressure.

Data analysis. The tape-recorded nerve activity was electronically filtered and fed into a window discriminator which selected action potentials on the basis of amplitude. The discriminator output was accumulated in an Ortec Model 4620 histogram computer. Poststimulus time histograms of nerve activity were obtained for 32 cardiac cycles. The upstroke of the R wave of the electrocardiogram was used to initiate the analysis of nerve activity, pressure, and flow data in 5-msec time increments. Frequency histograms, average isovolumetric pressure, and isobaric volume curves were traced out by a Honeywell 530 X-Y plotter.

Mechanical systole in the isovolumetrically contracting left ventricle was defined as the interval between the onset and termination of isovolumetrically developed ventricular pressure. In the isobarically contracting preparation, mechanical systole was taken as the interval between the onset and termination of the ejected volume signal. Nerve activity was averaged with respect to time during systole and diastole for each of the manipulated pressure levels. Data for each preparation were normalized by setting the maximum nerve activity recorded during systole and diastole as 100%. The data from all of the preparations were combined at each of the pressure steps utilizing these normalized values. Nerve activity at elevated pressures, both isovolumetric and isobaric, was compared to control values at 0 mm Hg. Significant differences in mean val-

ues from control were established using a Student's t distribution.

Results. Isobaric nerve activity was studied in eight nerve preparations in five mongrel dogs. Isovolumetric nerve activity was studied in six nerve preparations in six animals. Three of the fourteen small nerve fiber preparations were from animals in which isobaric and isovolumetric conditions were imposed alternately. The origin of the receptors which were stimulated by changes in left ventricular pressure or volume was confirmed by probing the epicardial surface of the left ventricle with a blunt insulated probe. Receptors were present throughout the left ventricle, and activation of a receptor by means of probing resulted in sustained nerve activity.

Representative nerve activity recorded from the third thoracic white ramus during isovolumetric contraction at 100 mm Hg peak systolic isovolumetric pressure is shown in Fig. 2A. Grouped systolic nerve activity was evident beginning with the onset of ventricular systole and through the period of ventricular pressure development. Minimal nerve activity was observed during ventricular diastole when pressures were at minimal levels.

Representative responses of afferent nerve activity during isobaric contraction are shown in Fig. 2B. Minimum values of ejected volume represent periods when ventricular diameter was maximum. Ejection of air by the contracting ventricle into the large reservoir of relatively high compliance and inertance produced the oscillations seen in the flow signal. In each case, ventricular contraction with concomitant reduction in ventricular diameter decreased the nerve activity from that seen in diastole. Delays due to conduction velocity (8–15 m/sec; 20-cm distance) and delays due to the transducer system are in the same direction and tend to partially cancel each other. Delays of the nerve activity relative to the transducer system are expected to be only 5–10 msec.

Poststimulus time histograms of afferent nerve activity recorded from R wave to R wave of the ECG for 32 cardiac cycles are shown in Fig. 3. Isovolumetric contraction produced increases in mean spike frequency occurring beneath the systolic pressure

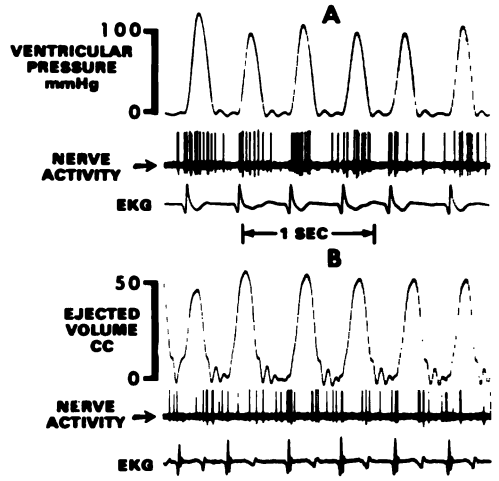
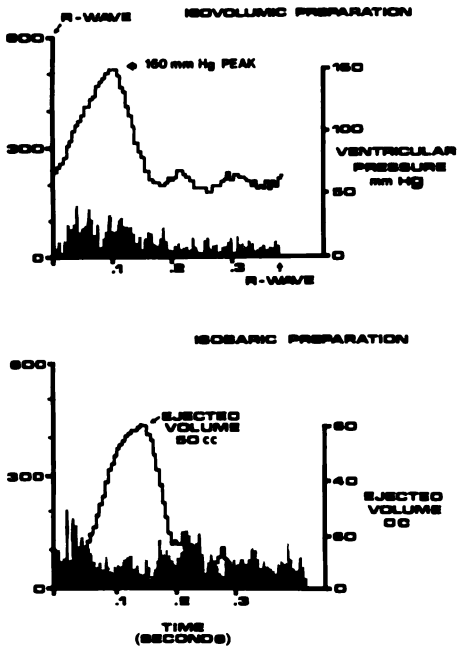


FIG. 2. (A) Effect of isovolumetric contraction on sympathetic afferent nerve activity. Peak systolic isovolumetric pressure at approximately 100 mm Hg. (B) Effect of isobaric contraction on sympathetic afferent nerve activity. Peak ejected volume at approximately 50 cm^3.

curve. Isobaric contractions produced increases in mean spike frequency occurring outside of isobaric systole.

Isobaric contraction resulted in no change in nerve activity during isobaric systole; however, return of the ventricle to diastolic dimensions resulted in increases in spike frequency. During isobaric studies, the most evident alterations in mean spike frequency occurred in late ventricular systole, early ventricular diastole, and during the early part of the ventricular systole associated with tension development in the myocardium.

Maximum discharge frequencies during isovolumetric contraction ranged from 87–169 impulses/beat during diastole. Maximum discharge frequencies during isobaric contraction ranged from 75–181 impulses/beat during systole and from 71–212 impulses/beat during diastole. Average nerve activities were plotted as percentages of maximum nerve activity accumulated for 32 cardiac cycles (Fig. 4). Mean discharge frequency and total nerve activity during isovolumetric systole responded to elevations in isovolumetric pressure. Nerve activity at peak systolic isovolumetric pressures of 100 and 150 mm Hg was significantly different ($P < 0.05$) from that of control taken as 0



G. 3. Poststimulus time histograms of spike frecy recorded for 32 cardiac cycles. Activity was ded during the R-R interval of the ECG. The lines represent average pressure (above) and flow w) tracings.

Hg. Diastolic nerve activity at a diaic pressure of 50 mm Hg was signifitly different from control.

sobaric left ventricular systole resulted in significant changes in nerve activity at isobaric pressure. Diastolic isobaric ve activity was significantly increased (P).05) by increases in isobaric pressure ventricular diameter. The average ted volumes at isobaric pressures of 0, 50, 75, and 100 mm Hg were 0, 40, 60, and 75 ml, respectively. Systolic nerve vity was independent of ejected volume he range of 0–80 ml. Diastolic nerve vity was significantly increased when ted volume increased from 0–80 ml.

iscussion. Sympathetic afferent nerve vity of left ventricular origin responded ıcremental increases in peak systolic isoımetric pressure with significant inıses in average nerve activity. These data firm earlier evidence by Hess *et al.* (8) of ısure-sensitive left ventricular mechanoptors with fibers traversing the left upthoracic white rami communicantes. In the present study, during isovolumetric conditions, nerve activity was influenced to a greater extent by the development of systolic pressure than by diastolic pressure.

Elevation of isobaric pressure during isobaric systole resulted in no significant alterations in the percentage of maximum systolic nerve activity. Since intraventricular pressure remained constant during isobaric systolic contraction, ventricular contraction alone, in the absence of pressure changes, was not capable of altering afferent nerve activity.

Significant changes in maximum nerve activity under isobaric conditions were evident during the period of ventricular diastole at elevated isobaric pressures. Large alterations in diastolic ventricular dimensions appeared to produce the most significant response in these cardiac afferent fibers. Cardiac sympathetic afferent receptors may be similar to ventricular receptors with vagal

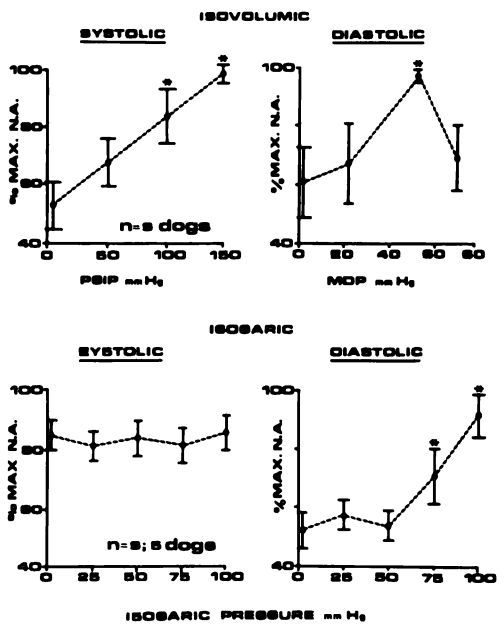


FIG. 4. Comparison of isovolumetric and isobaric average nerve activity plotted as percentage of maximum nerve activity during systole and diastole. Isovolumetric systolic nerve activity is plotted against peak systolic isovolumetric pressure (PSIP) in mm Hg. Isovolumetric diastolic nerve activity is plotted against mean diastolic pressure (MDP) in mm Hg. Systolic and diastolic isobaric nerve activity are plotted against isobaric pressure in mm Hg. Vertical bars represent ± standard error.

afferents which respond to changes in ventricular pressure and/or volume, as described by Coleridge *et al.* (14).

An isobaric method has been described previously by Monroe *et al.* (9). A modification of this method was used in the present study. The unique experimental apparatus employed in the present study related cardiac afferent nerve activity to changes in left ventricular mechanics in a manner not previously described. Previous studies of the isovolumetrically contracting ventricle (8) may have selected solely for sympathetic afferent nerves responding to changes in developed pressure and did not demonstrate the full capabilities of the afferent response.

The afferent nerve activity described here may initiate reflexes, as suggested by Schwartz *et al.* (10). Activation of this reflex produced an increase in sympathetic afferent activity resulting in a rise in arterial pressure (11), an increase in myocardial contractility (12), and an acceleration of heart rate (13), accompanied by a decrease in cardiac vagal activity (10).

Summary. We conclude that the increase in isovolumetrically developed intraventricular pressure is associated with an enhanced activity of cardiac sympathetic afferents traversing the left upper thoracic white rami communicantes, as reported by Hess *et al.* (8). Moreover, the sympathetic afferent nerve fibers respond to alteration in myocardial muscle length occurring in i diastole and thus respond to a displa stimulus as well as to a pressure stim

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THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 505-507 (1977)

ated Expression of T-Antigen in Skin Fibroblasts from Individuals with ynetic Anomalies Infected *in Vitro* with Simian or Human Papovaviruses[1] (39704)

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l groups with elevated cancer risk :ased expression of viral genetic following *in vitro* infection with povavirus 40 (SV40) include Fan- ıia (1-5), Down's syndrome (3, 4, somy 18 (3, 6), Klinefelter's syn- 3), and a leukemia-prone family laboratory has confirmed and ex- is positive correlation between the of SV40-induced T antigen or ıation in infected human skin fibro- d some types of increased cancer e cell donor (manuscripts submit- ıblication). We have further shown 0 T antigen expression by fibro- ım normal individuals showed no t differences among various age, :hnic groups examined (submitted :ation).

reports have shown serological re- between the T antigens (10) and ıcleic acid homology between the of SV40 and the human papovavi- (11). BKV(MM), isolated from a ıor and the urine of a patient with Aldrich syndrome (12), has also wn to complement an SV40 tem- sensitive mutant defective in an ınction (13), possibly linked to T- xpression (14, 15). In view of these general similarities between sim- uman papovavirus (10), we ques- ıether a human papovavirus might ıit elevated T-antigen expression in oblasts from high cancer risk

ls and methods. Human skin fibroblast cultures were established from punch biopsies and propagated as reported (16). BKV(MM) was propagated and assayed on human embryonic kidney cells as reported previously (13). The preparation employed contained 1×10^6 tissue culture infectious doses ($TCID_{50}$)/ml assayed on human embryonic kidney cells by limiting dilution methods. SV40 was propagated and assayed on African green monkey kidney (17). The stock contained 2×10^8 PFU/ml.

Human cells were infected with 100 PFU/cell of SV40 or 5 $TCID_{50}$/ml of BKV(MM), as reported previously (18). At 72 hr after infection, indirect immunofluorescent assays for T antigen were performed using hamster sera raised against SV40 T antigen (16). From previous preliminary studies, it was known that these reagents did not stain uninfected human skin cells in over 200 cell strains examined.

The weighted mean proportion (p_w) of T-antigen-containing cells was calculated from three replicate petri plates per determination (18). On each plate, 100 microscopic fields (averaging $3-5 \times 10^3$ cells) were examined for T-antigen expression. Each replicate plate was randomly encoded before staining to prevent operator bias; codes were not broken until the conclusion of the assay.

Results. Figure 1 shows that T-antigen expression by eight human fibroblast strains tended to be similar for SV40 and BKV(MM). For both viruses, the four normal cell strains expressed T antigen less frequently than cell strains from the three clinical groups at high cancer risk. The standard errors were relatively larger for BKV(MM)-infected cells than for SV40-infected cells in every cell strain. This may reflect the less intense fluorescence generally observed in BKV(MM)-infected cells.

ork was supported in part by the National itute Contract No. NO1-CP-43216.

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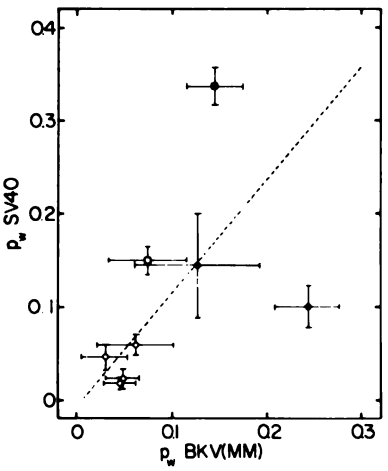

FIG. 1. T-antigen expression by BKV(MM)-infected and SV40-infected human skin fibroblasts. Weighted mean proportions (p_w) for each cell strain and virus examined were calculated as described (18). Each of four normal human fibroblast strains are shown as open circles, each of two strains from Fanconi anemia patients are shown as closed circles, and single strains from patients with Turner's syndrome and Klinefelter's syndrome are shown by open and closed squares, respectively. Bars indicate one standard error about the mean value. The dashed line indicates the nonparametric regression line.

To avoid assumptions about the underlying mathematical distribution of the data, nonparametric statistical analyses were performed. Employing Spearman's rank correlation method (19), data from BKV(MM)-infected cells correlated with results from SV40-infected cells ($r_S = 0.76$; $P_{r_S=0} < 0.05$). Nonparametric linear regression analysis was employed using the methods of Theil (20). Assuming that BKV(MM) and SV40 behaved identically (despite apparent differences in the multiplicity of infection) in all cell strains tested, the expectation is that the regression line of the data in Fig. 1 has a slope of unity and passes through the origin. In fact, the slope estimator possessed a value of 1.27, which was not significantly different from unity ($P > 0.05$). The regression line passes very close to the origin in Fig. 1.

Discussion. The observations of Todaro and his associates that fibroblasts from patients with several diseases associated with increased cancer risk have increased frequency of SV40 transformation and T-antigen expression have been of great interest to oncologists (1, 2, 6). These experiments with SV40, however, require high multiplicities of virus (18), and the significance of increased susceptibility of human cells to infection *in vitro* by a monkey virus has remained obscure. The fact that generally similar susceptibilities are found with a human papovavirus at apparently lower multiplicities provides the previously missing rationale for both of these observations.

Klein (21) has recently suggested that some forms of viral carcinogenesis may require specific genetic changes for development of neoplasia *in vivo* and that chromosomal abnormalities may play a critical role in oncogenesis in man by Epstein–Barr virus. The present findings that T-antigen expression is elevated in human papovavirus-infected fibroblasts from patients with Fanconi anemia, Turner's syndrome, and Klinefelter's syndrome is consistent with this view of viral oncogenesis.

The present results also permit analysis of the relative infectivity of SV40 and BKV(MM) for human cells. It is known that the level of T-antigen expression by SV40-infected cells is multiplicity dependent (18, 22). Since BKV(MM) can be titrated by a limiting dilution T-antigen assay (23), T-antigen expression is probably multiplicity dependent for this virus also. Thus, the level of T-antigen expression is a measure of the number of virus particles infectious for a given cell strain. If it is assumed that 1 $TCID_{50}$ of BKV(MM) consists of a single infectious particle, then BKV(MM) is approximately 20 times more infectious for human cells than SV40. This is derived from the fact that 5 $TCID_{50}$/cell of BKV(MM) (titrated on human cells) induced approximately the same level of T-antigen expression as 100 PFU/cell of SV40 (titrated on monkey cells). This quantitative estimate is consistent with qualitative evidence of the host cell preferences of these viruses (10).

Summary. Human skin fibroblasts were infected with human papovavirus (BK type) or simian papovavirus 40 (SV40) at multiplicities of infection which induced similar frequencies of T-antigen-containing cells at 72 hr after infection. Both viruses exhibited a higher rate of T-antigen expression in cells

from genetically abnormal individuals with Fanconi anemia, Turner's syndrome, or Klinefelter's syndrome than in cells from normal individuals.

The authors wish to express their gratitude to Mark Gunnell, Thomas Archer, Elizabeth Alexander, and Carole Yee for their excellent technical assistance, and Celeste Martin for her patient secretarial assistance.

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Received October 18, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 508-512 (1977)

Ribonucleoside and Nucleotide Components of Normal Human Blood Lymphocytes and Platelets (39705)

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Introduction. The analysis of intracellular nucleotide levels has contributed to the understanding of several physiologic and biologic regulatory processes in various cells and tissues (1-3). Major attention has been directed to studies of variation of the cyclic nucleotides, cAMP and cGMP (4, 5), with relatively little information available concerning the levels of the ribonucleoside or the 5'-mono-, 5'-di-, and 5'-triphosphates. Perhaps one reason for the "neglect" is the rather complicated procedures necessary for the detection and quantitation of the noncyclic nucleotides. Advances in the techniques of high pressure liquid chromatography have tended to minimize these complications.

The present studies were designed to identify and quantitate the nucleosides and noncyclic nucleotides present in human blood platelets and lymphocytes. Since the lymphocytes can be divided into two basic populations (T and B cells) that differ according to many criteria including ontogeny, surface receptors, immunologic function (6), and adherence to nylon wool columns (7), chromatographic profiles of the two populations were determined.

Materials and methods. Platelet isolation. Blood (50 ml) from healthy adult volunteers was collected into syringes containing 4.5 ml of heparin (1000 USP units/ml). The blood was centrifuged at 300*g*, the platelet-rich plasma (PRP) was carefully removed, and the number of platelets was determined by a thrombocounter (Coulter Electronics, Inc., Hialeah, Fla.). The platelets were sedimented by centrifugation at 1800*g*, frozen immediately in an alcohol-dry ice bath, and stored at −80°.

Lymphocyte isolation. The platelet-poor plasma (PPP) was added back to the sedimented blood. Tubes consisting of 10 ml of blood and 10 ml of 0.15 *M* NaCl underlayered with 15 ml of Lymphoprep (Gallerd-Schlesinger Chem, Carle Place, N.Y.) were centrifuged at 400*g* at the interface, 40 min, 22° (7). The layer containing lymphocytes was removed and centrifuged, and the sedimented cells were washed 2× with Hank's balanced salt solution (HBSS, Flow Laboratories, Rockville, Md.) and resuspended in 2-ml volumes of BME-FCS consisting of Basal Medium Eagle (BME, Flow Laboratory, Rockville, Md.) plus 5% heat-inactivated fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.). Viabilities were determined by exclusion of 0.4% trypan blue (Grand Island Biological Company, Grand Island, N.Y.).

Lymphocyte subpopulations. Nylon wool columns were prepared as described in Trizio and Cudkowicz (8) with all operations performed at 37°. Two milliliters of lymphocyte preparation were added to the column and incubated for 45 min. "Effluent" cells were obtained by passage of 25 ml of BME-FCS through the column. The column was then washed with 100 ml of media and "eluted" cells were obtained by pressing the wool with forceps.

Erythrocyte rosettes. Nonimmune (E) and complement-binding (EAC) rosettes were prepared as previously described (9). Sheep red blood cells (SRBC) were obtained each week from Lee Laboratories, Grayson, Ga. For E rosettes, SRBC were washed, and a 0.5% suspension was made in one part HBSS and one part heat-inactivated, SRBC-absorbed, fetal calf serum. Lymphocytes (1×10^6/0.25 ml) and SRBC (0.25 ml) were incubated for 5 min at 37°, centrifuged at 200*g* for 5 min at 22°, and incubated overnight at refrigerator temperatures. For enumeration of rosettes, the cells

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ISSN 0037-9727

tly suspended by rotating the tube, μl of cells were mixed with 50 μl of thylene blue. For EAC rosettes, tivated hemolysin (Difco, Detroit, .02 ml) at one-half the minimum ting dose was added to 10 ml of a spension of SRBC, and incubated r 30 min. Mouse serum as comple- rce (0.2 ml) was added to 1.8 ml of uspension, incubated for 30 min at washed 3× in HBSS. The EAC n indicator cells (0.25 ml) were l with lymphocytes (1 × 10^6/0.25 15 min at 37° and centrifuged at °, for 5 min. The cells were sus- n a vortex, and 200 μl of cells were ith 50 μl of 10% methylene blue ted in a hemocytometer. A mini- 300 cells were counted by hemocy- with those containing three or more SRBC being scored as rosettes.

side/nucleotide extraction. Nucleo- e extracted by adding 1 ml of cold A/5 × 10^9 platelets or 1 ml 12% < 10^8 lymphocytes as described by *t al.* (1). Proteins were pelleted by ation at 1800*g* for 5 min at 4° and natant was removed. The pH was up to approximately 6 by four to actions with cold-water-saturated her. The ether was bubbled off and les were lyophilized and stored at fore the run, samples were recon- n 0.15 ml of H_2O (lymphocytes) or H_2O (platelets). Volumes of 50 μl ytes) or 25 μl (platelets) were the column. A column extract re- m a minimum of approximately 1 nphocytes of 6 × 10^8 platelets.

chromatography. The general pro- of separation and analysis have cribed by Brown (10). We used a lmer (Norwalk, Conn.) LCS 1220 LCS 55 detector operated at 260 column was partisil sax 10/25. The hase consisted of linear gradient of $NH_4H_2PO_4$, pH 2.8, to 0.5 *M* O_4, pH 4.8; 5%/min. The flow rate /min at 40°. The Perkin-Elmer 56 was utilized and the quantitations ed by the PEP-1 data system which l the response factors (RF) of un- mple peaks with standard peaks of

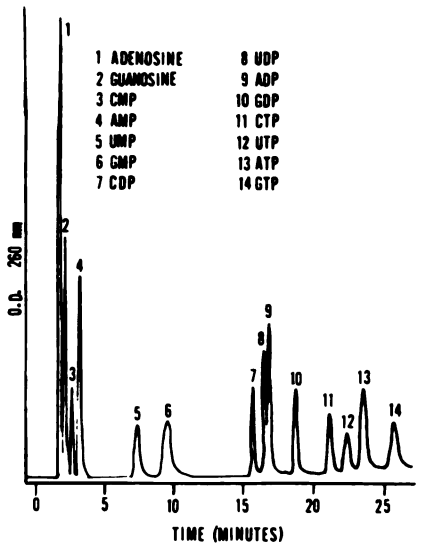

FIG. 1. Standard mixture of ribonucleosides and nucleotides (each 1-2 n*M* in a 10-μl volume loaded onto the column). Retention times in minutes are as follows: (1) adenosine, 1.52; (2) guanosine, 1.86; (3) CMP, 2.88; (4) AMP, 2.93; (5) UMP, 7.20; (6) GMP, 9.42; (7) CDP, 15.62; (8) UDP, 16.56; (9) ADP, 16.84; (10) GDP, 18.78; (11) CTP, 21.22; (12) UTP, 22.48; (13) ATP, 23.69; (14) GTP, 25.89.

known concentrations.

Results. Figure 1 shows the retention times of a standard mixture of ribonucleosides and 5′-mono-, 5′-di-, and 5′-triphosphates under normal operating conditions. Standards were run before each experiment and the times proved to be very reproducible. Figure 2 shows a sample chromatogram of (A) platelets and (B) nylon wool effluent cells. Table I shows the concentrations (nmole/10^9 platelets) of human platelet nucleosides and nucleotides. The ratio of ATP/ADP was 1.6:1, in agreement with others (1, 11). The GTP:GDP ratio was 1:1, also in agreement with others (1, 11). It should be noted, however, that it was possible to identify components not previously reported. Thus, AMP, GMP, CTP, guanosine, and adenosine can be easily quantitated in addition to the previously studied 5′-di- and 5′-triphosphates.

Peripheral blood lymphocytes of normal adults were separated into two populations according to differential adherence to nylon wool columns. The efficiency of the nylon wool column was determined and presented

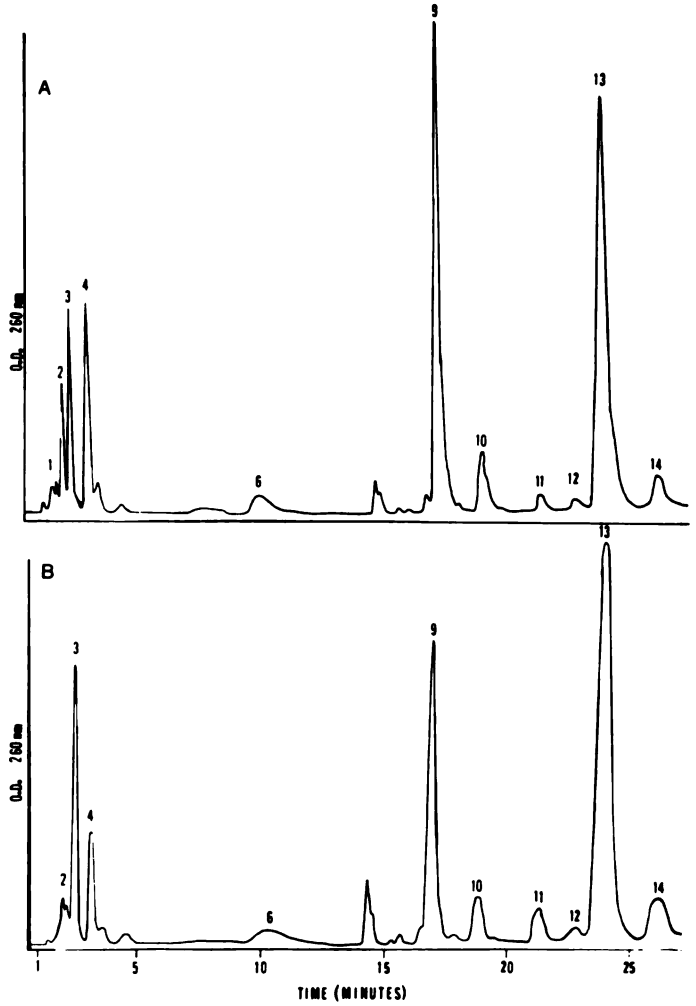

FIG. 2. A. Platelet sample. Following lyophilization, residue was redissolved in 0.3 ml of cold deionized water and 25 μl was loaded on column. B. Effluent cell sample. Residue was redissolved in 0.15 ml of cold deionized water and 50 μl was loaded on column. The peaks can be identified by referring to the legend for Fig. 1.

in Table II. E rosettes were used as a measure of T lymphocytes; EAC rosettes were used as a measure of B lymphocytes (9). This procedure removed about half of the EAC-rosette-forming cells from the effluent fraction relative to whole blood (18.9 vs 9.7%) and reduced the number of E-forming cells in eluted fraction by about 2/3 (50.7 vs 15.2%). The T cells were always 95% viable. The viability of the B cells averaged 85%.

Table III shows the nucleotide concentrations in the effluent and eluted cells. The low numbers of B cells in peripheral blood forced a pooling of the samples. The ATP/ADP ratio in the effluent cells was 3.3 whereas in the eluted cells it was 1.5. The GTP/GDP ratio in the effluent cells was 1.8, while that of the eluted cells was 0.5. In addition to the adenosine and guanosine nucleotides, it was possible to quantitatively estimate the concentration of CMP, CTP, and UTP in these cells.

Discussion. High-pressure liquid chromatography employed in the present investigation has provided a method to identify and quantitate nucleotide components previously undetected even by similar techniques. Scholar *et al.* (1) have shown the presence of adenosine and guanosine 5'-di-

and 5′-triphosphates in platelets; however, AMP, GMP, CMP, GTP, UTP, guanosine, and adenosine were not reported. Goetz *et al.* (11) have quantitated AMP and GMP in platelets, but were unable to separate GDP from UTP.

Nylon wool columns provide a quick way to partially purify E- and EAC-forming cells. The data suggest a different nucleotide profile in effluent and eluted cells from nylon wool with a higher concentration of the triphosphates present in the effluent components. It should be remembered, however, that the effluent cell data represent the average of nine individual experiments, whereas the eluted cell data represent two pools of cells. Also, the viability of the effluent cells, as determined by trypan blue exclusion, was excellent (>95%); the viability of the eluted cells averaged 85% (ranged from 82–90%).

The large majority of peripheral blood lymphocytes are T cells. Although the concentrations of ATP, CTP, and UTP noted in the effluent cells compared favorably to those reported by Scholar *et al.*, (1), we found higher concentrations of mono- and diphosphates. The reason for this variation is not clear, although it most probably is due to column differences. It should be noted that varying concentrations of the adenosine and guanosine diphosphate have been reported by several laboratories (1, 11, 12).

Experimental evidence has shown that the normal nucleotide levels of certain types can be altered through biochemical or pharmacological mechanisms (12). The role of the adenylates as regulatory effectors has been well-documented (14). Nucleotides (particularly cyclic nucleotides) have been implicated in T cell–B cell interactions, E rosetting, and mitogen stimulation (15, 16). Correlations between disease states and subtle changes in nucleotide composition have been proposed (1, 2). It may be that the establishment of standard values of nucleotide concentrations in normal blood elements will aid in recognizing variations in these pools resulting from pathological states.

It is obvious that one cannot utilize the procedures described in this report for an analysis of cyclic nucleotides in concentrations less than approximately 200 pmole. If the samples are concentrated to a point that

TABLE I. RIBONUCLEOSIDE AND NUCLEOTIDE CONCENTRATIONS IN HUMAN PLATELETS (NMOLE/10^9 PLATELETS).

Component	$\bar{X}$[a]	SE
AMP	4.904	0.431
ADP	19.281	1.341
ATP	31.887	2.272
GMP	2.677	0.518
GDP	4.565	0.373
GTP	4.892	0.474
CMP	15.311	0.233
CTP	1.410	0.285
UTP	2.040	0.256
Guanosine	1.305	0.884
Adenosine	0.165	0.019

[a] Mean ± standard error of the mean of nine samples.

TABLE III. NUCLEOTIDE CONCENTRATIONS (NMOLE/10^7 CELLS) OF LYMPHOCYTES FROM NYLON WOOL.

	Effluent cells[a]		Eluted cells[b]	
Component	$\bar{X}$	SE	$\bar{X}$	SE
AMP	0.915	0.223	3.689	0.911
ADP	2.998	0.503	2.368	1.111
ATP	9.837	1.201	3.677	1.411
GMP	0.670	0.201	0.362	0.050
GDP	0.977	0.138	1.221	0.362
GTP	1.767	0.304	0.589	0.446
CMP	1.200	0.116	5.889	3.775
CTP	1.200	0.116	0.485	0.063
UTP	0.817	0.182	0.178	0.178
Guanosine	0.236	0.050		

[a] Average based on nine samples individually run.
[b] Average based on two pools of samples (pool 1 had six samples; pool 2 had three samples).

TABLE II. SEPARATION OF LYMPHOCYTE SUBPOPULATIONS BY NYLON WOOL.

	Recovery from column[a] (%)	E (%)	EAC (%)	E/EAC	EAC/E
Before nylon wool	–	50.7 ± 5.2[b]	18.9 ± 2.9	2.7	0.4
Effluent cells	56.3	56.9 ± 6.9	9.7 ± 2.6	5.7	0.2
Eluted cells	10.5	15.2 ± 6.4	30.5 ± 7.7	0.50	2.0

[a] Percentage of total cells placed on wool.
[b] Mean ± standard deviation.

cAMP or cGMP would be detectable, the other nucleotides would overlap. Attempts are underway to develop a method to convert nucleotides (other than the cyclic) to nucleosides to offset the above problem and this will be reported at a later date.

Summary. Ribonucleoside and nucleotide profiles of human peripheral blood platelets, nylon wool effluent, and nylon wool eluted cells were obtained by high pressure liquid chromatography. AMP, ADP, ATP, GMP, GDP, GTP, CMP, CTP, and UTP components were present in all cell types. Guanosine was found in platelets and effluent cells. Adenosine was detected in platelets only. The use of high pressure liquid chromatography provides a rapid, reproducible means for analyses of nucleotides in blood elements.

We would like to acknowledge the expert technical assistance of Mrs. Betty Nager.

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Received June 28, 1976. P.S.E.B.M. 1977, Vol. 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 513-516 (1977)

ı Kinase Activity of Isolated Rat Adipocytes as Related to Cell Size[1] (39706)

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uction. Enlarged adipocytes from tter rats present a number of metaerations when compared to smaller from leaner younger rats (1-5). the most significant findings, large es (mean volume, 400-600 pl) a rate of basal lipolysis which is 3- to reater than that of smaller fat cells pl) (2-4); in addition, large adipoesent a rate of glyceride synthesis ıcose which is three to four times than that of the smaller cells (3-5). indings have indicated an acceleracellular turnover of glyceride in ırged cells (5), the purpose and sm(s) of which are not known at

ıtion of the hormone-sensitive li- 7) in adipocytes follows stimulation l cyclase, formation of cyclic AMP, sequent activation of cyclic AMP- nt protein kinase. Hartman *et al.* reported that norepinephrine-actilenyl cyclase activity in adipocytes, d on a per-cell basis, was unaffected size. Cooper and Gregerman (8) ıat both basal and epinephrine-actidenyl cyclase activities in isolated es declined progressively and signifn rats from 2 to 24 months of age. *al.* (9) have shown that, in intact fat e norepinephrine-stimulated adenyl ıctivity was similar in cells from 5- to old and 10- to 12-week-old rats, but kedly decreased in cells from 18- to -old rats.

authors also found an increase in diesterase activity with aging and/ ging cell size in the rat. No values could be found in the literature for unstimulated cyclic AMP content of fat cells as related to cell size.

Since these reported changes could not readily explain the increased basal rate of lipolysis in enlarged fat cells, the possibility was explored that the cyclic AMP content and the adipocyte protein kinase activity may be altered with enlarging fat cell size in the rat, in a manner more closely related to the changing lipolytic rate. In the present report we have compared basal, cyclic AMP-dependent, and cyclic GMP-dependent protein kinase activities in adipocytes differing in mean cell volume, both on the basis of protein content and also on a per cell basis. The cellular content of cyclic AMP was also compared.

Materials and methods. Animals. Male Wistar rats (Royalhart Laboratory, New Hampton, N.Y.), fed Purina laboratory chow ad libitum since weaning, were housed in a temperature-controlled (23°) room and exposed to 12-hr intervals of light and darkness. Water was available at all times. The animals were sacrificed by cervical dislocation at 9-10 A M in the fed state.

Conditions of incubation and analytical techniques. At sacrifice, the epididymal fat pads were removed and weighed; after mincing, they were incubated at 37° in Krebs-Ringer bicarbonate medium (KRB), at pH 7.4, with collagenase from *Cl. Histolyticum* (Worthington, 5-10 mg/g adipose tissue), as described by Rodbell (10). The fat cells were then washed four times and resuspended in fresh medium by techniques previously employed (4). The resuspended fat cells were incubated for 15 min at 37° in a Dubnoff metabolic shaker at 80 strokes/min. Three-milliliter aliquots of the cell suspension were transferred to a plastic centrifuge tube, kept at 0°, and sonicated for 10 sec. Additional aliquots of the cell suspensions were removed for the determination of lipid content and for optical sizing of the cell

work was supported in part by Grants AM-M-17403, and HL-15696 from the United blic Health Service.

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1977 by the Society for Experimental Biology and Medicine
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ISSN 0037-9727

diameters; from these, the fat cell number was calculated (11).

Protein kinase activity was determined in the infranatant obtained after centrifuging the sonicated cells for 10 min at 30,000*g*. The standard assay system for cyclic AMP-dependent and cyclic GMP-dependent protein kinases was essentially the same as described elsewhere (12), except that the isolated stimulatory modulator (13) of cyclic GMP-dependent protein kinase replaced the crude protein kinase modulator. The incubation mixture contained, in a final volume of 0.2 ml: potassium phosphate buffer, 10 μmole, pH 7.0; arginine-rich histone (HA, Worthington), 40 μg; $MgCl_2$, 2 μmole; [γ-^{32}P]ATP (New England Nuclear), 1 nmole, containing about 1.2×10^6 cpm; stimulatory modulator from bovine heart (45 μg); with or without cyclic AMP or cyclic GMP, 80 pmole as indicated. The reaction was carried out for 10 min at 30°. One unit of the enzyme is defined as that amount of enzyme that transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered histone under the assay conditions. The protein content of the infranatant was determined by the method of Lowry (14). Protein kinase activity was expressed as units of activity per milligram of cell protein and also per fat cell. Cyclic AMP was determined by the method of Kuo and Greengard (15) and expressed as pmole/10^6 fat cells.

Results. The determinations were carried out in two groups of rats: group A, lean rats (6–7 weeks in age) weighing between 150 and 170 g; and group B, older fatter rats (5–6 months in age) weighing between 450 and 550 g. The epididymal fat pads of five to six animals of group A were pooled to obtain sufficient adipocytes in a single experiment, whereas in group B the pads of only one animal were used. The mean fat cell volume of group A rats was 58 pl, that of group B rats was 371 pl. Table I shows that the larger adipocytes from group B rats contained 2.3 times more protein per fat cell than the smaller adipocytes from group A rats. It also shows that basal protein kinase activity was significantly greater for group B rats than group A rats, whether the activity was expressed as units/milligram of protein (230 vs 168, $p < 0.02$), or as units/10^6 fat cells (422 vs 131, $p < 0.001$). Similarly, the cyclic AMP-dependent protein kinase activity was also greater in the large adipocytes of group B rats than the small adipocytes of group A ($p < 0.005$), independently of the unit of expression. No significant protein kinase activity specific for cyclic GMP was found in extracts of fat cells from either group. This enzyme activity, however, has been shown to become evident upon further purification (12). The cyclic AMP-stimulated protein kinase activity was markedly and significantly greater ($p < 0.001$) than basal kinase activity in both types of cells. When expressed as relative increase over the basal levels, the enzyme activity was 252% of basal values in group A, and 270% of basal in group B. Thus, even though cyclic AMP-dependent protein kinase activity was markedly greater in the large fat cells (46% higher on protein basis, or 246% higher on per cell basis) when compared to small cells, the relative stimulation by cyclic AMP was similar in adipocytes from both groups. Table II shows that the basal cyclic AMP levels, expressed on a per-cell basis, were significantly greater ($p < 0.025$) in the larger adipocytes from group B than in the smaller adipocytes from group A. It is of

TABLE I. PROTEIN KINASE ACTIVITY OF ADIPOCYTES OF DIFFERENT SIZE FROM TWO GROUPS OF RATS.

			Protein Kinase Activity					
	Mean fat cell Volume	Protein/fat cell	Basal	+Cyclic GMP	+Cyclic AMP	Basal	+Cyclic GMP	+Cyclic AMP
Group*	(pl)	(ng)	(U/mg of protein)			(U/10^6 fat cells)		
A (n = 6)	58 ± 3	0.78 ± 0.05	168 ± 11	172 ± 9	424 ± 16	131 ± 13	135 ± 12	331 ± 22
B (n = 7)	371 ± 23	1.82 ± 0.12	230 ± 18	243 ± 18	621 ± 48	422 ± 55	445 ± 53	1144 ± 150
p†	<0.001	<0.001	<0.02	<0.01	<0.005	<0.001	<0.001	<0.001

* Group A rats were 6–7 weeks old, body weight 150–170 g; Group B rats were 5–6 months old, body weight 450–550 g; n is the number of experiments in each group of animals. Figures are given as means ± SE.

† p represents the statistical significance of the difference between means of respective measurements in group A and group B rats.

TABLE II. CYCLIC AMP CONTENT OF ADIPOCYTES OF DIFFERENT SIZE FROM TWO GROUPS OF RATS.

Group[a]	Mean fat cell volume (pl)	Cyclic AMP (pmole/10^6 fat cells)
A	64 ± 3	10.36 ± 1.13
B	439 ± 29	27.25 ± 4.98
p[b]	<0.001	<0.025

[a] Group A rats were 6–7 weeks old, body weight 150–170 g; Group B rats were 5–6 months old, body weight 450–550 g. Figures given are means ± SE.

[b] p represents the statistical significance of the difference between means of respective measurements in group A and group B rats.

note that the magnitude of the difference in cyclic AMP between mean values of large and small fat cells (2.63 ×) per cell was approximately similar to the difference in basal protein kinase activity per cell (3.22 ×) in the two groups.

Discussion. Large adipocytes from old fat rats differ in several morphological and metabolic parameters when compared to smaller adipocytes from young lean rats (1–5, 11). The present study provides an additional instance of a marked difference in an important regulatory system between small and large adipocytes.

Protein kinase in fat cells is known to mediate the effect of cyclic AMP on lipase activation (6, 7). Subsequent to this enzyme activation, triglycerides are hydrolyzed and both glycerol release and free fatty acid mobilization ensue. The present findings of a 3-fold greater cyclic AMP content and a 3- to 4-fold higher basal protein kinase activity in large adipocytes are consonant with the corresponding greater rate of basal lipolysis in these cells as compared to that of smaller adipocytes from younger leaner rats (2–4).

The observed differences between small and large fat cells in basal and cyclic AMP-stimulated protein kinase activity are more pronounced when the results are expressed on the basis of fat cell number than when they are expressed on the basis of cellular protein. This is probably due to a greater protein content in the enlarged adipocytes; in the present study, approximately 2.3 times more cellular protein is found in enlarged fat cells than in small fat cells. Thus, an enlargement of fat cell size leads to an increase not only in surface area and volume but also in the amounts of cellular protein.

It is of interest that other investigators have reported, with aging and/or enlarging cell size in the rat, no change or a decrease in basal and epinephrine-stimulated adenylate cyclase (2, 8, 9) and also an increase in phosphodiesterase activity (8) of the adipocytes. Neither of these enzymatic changes could be readily reconciled with the greater rate of basal lipolysis in the large fat cells. The present study, by demonstrating that larger adipocytes contain greater amounts of cyclic AMP and present a higher basal protein kinase activity, suggest that these two cellular parameters may be interrelated and possibly responsible for the greater baseline lipolytic activity observed in the large as compared to the smaller fat cells. Addition of cyclic AMP to the cellular extract in this study stimulated protein kinase activity to a similar extent in both small and large fat cells. These findings may also be consonant with a similar degree of lipolytic stimulation exerted by lipolytic hormones on fat cells of different size (2, 15, 16), although one must be cautious in attempting to correlate enzyme activation with overall metabolic responses such as lipolysis (9).

Summary. We have studied cellular cyclic AMP content and protein kinase activity in extracts of small and large adipocytes from two groups of rats differing in age and body weight. The data, expressed on a per cell basis, show that large adipocytes from larger rats present a significantly greater ($p < 0.02$) content of cyclic AMP and a 3-fold greater ($p < 0.001$) level of basal protein kinase activity than small adipocytes. Cyclic AMP (0.4 μM) stimulated the cyclic AMP-dependent protein kinase activity to a similar degree (about 3-fold) in both types of cells. No significant amount of cyclic GMP-dependent protein kinase activity was detected in extracts of adipocytes from either group of rats.

The increased cellular content of cyclic AMP and the elevated protein kinase activity in large adipocytes are probably responsible for the greater basal rate of lipolysis observed in these cells.

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Received October 11, 1976. P.S.E.B.M. 1977, Vol. 154.

F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 517-521 (1977)

ent Stabilization of Rat Liver Lysosomes by Cytosol and Serum Proteins[1] (39707)

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imes *in vivo* are surrounded by a nic milieu which may affect the :s and functions of this organelle. :ractions between this subcellular ind the cytoplasm are not well un- and need to be characterized in assess the physiological role of lyso- nteractions between lysosomes and nic constituents might be detected uring the distribution of lysosomal in soluble and particulate compart- fractions after incubation with var- chemicals. In such studies, lyso- ispended in isotonic sucrose have ubated with a large number of sub- 1). Many biochemicals have been :d as labilizers (e.g., vitamin A) or :s (e.g., cortisone) if they increased ased the distribution of lysosomal into the soluble fraction. The labi- d stabilizers are thought to disrupt :t, respectively, the lysosomal mem- he present paper reports that rat ible proteins (cytosol), as well as oteins, cause soluble lysosomal en- cosediment with cellular granules Although these protein solutions o stabilize lysosomes, in actuality iance interactions between lysoso- ymes and cellular particles by a sm other than stabilization of lyso- embranes.

als and methods. Subcellular parti- taining lysosomes were prepared from livers of male Holtzman rats (300 to 350 g) by homogenizing the sample in a tissue grinder with 0.25 *M* sucrose containing 0.01 *M* Tris-HCl buffer, pH 7.2, at a ratio of 1 g of liver:9 ml of sucrose solution. The centrifugation scheme for obtaining the large granular fraction (LGF), the fraction containing lysosomes, has been described by Weissmann (2). At each washing step, the LGF was suspended by directing a stream of buffered isotonic sucrose at the precipitate (3). The final protein concentration of the fraction was 2.4-4.0 mg/ml. To determine the total activity of β-glucuronidase, a sample of the large granular suspension was mixed with an equal volume of Triton X-100 [0.2% (v/v)], incubated at 37° for 30 min, and centrifuged at 20,000*g* for 20 min, and the supernatant fraction was assayed for enzyme activity.

To obtain the soluble contents (lysate) and sedimentable membranes of the LGF, the sedimented LGF was suspended and homogenized in distilled water and allowed to stand for 30 min at 4°. The osmotically shocked particles were then centrifuged at 48,000*g* for 80 min. The soluble portion (hypotonic lysate) was dialyzed against 1 m*M* Tris-HCl buffer, pH 7.2, for 12 hr at 4°; this portion contained 60-80% of the LGF β-glucuronidase activity. The sedimentable portion or membranes of the LGF, obtained after the hypotonic treatment, were homogenized into a buffered, isotonic sucrose solution.

For preparation of cytosol, liver samples were homogenized with 4 or 9 volumes of 0.25 *M* sucrose containing 0.01 *M* Tris-HCl, pH 7.2. Homogenates were centrifuged at 48,000*g* for 80 min and the supernatant (cytosol) fraction was decanted and stored at −20°. In some instances, livers were perfused with NaCl (0.9%) prior to homogenization and cytosol was dialyzed

ork was supported in part by NSF Grant and NIH Grant CA-13715; B.E.B. had a ginia Fellowship.

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977 by the Society for Experimental Biology and Medicine ved.

ISSN 0037-9727

for 48 hr at 4° against the homogenizing buffer. To prepare boiled cytosol, cytosol was placed in a test tube and immersed in boiling water for 30 min. The flocculent precipitate was suspended by homogenization.

β-Glucuronidase activity was measured according to the method of Talalay *et al.* (4). Acid deoxyribonuclease activity was assayed according to the method of Gianetto and de Duve (5). Malic dehydrogenase activity was measured by the method of Siegel and Bing (6). Catalase activity was measured by the method of Beers and Sizer (7). The Biuret method (8) or the method of Lowry *et al.* (9) was used to determine protein concentrations. In both methods, bovine serum albumin (Fraction V) was used as the standard. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), except as noted. Human γ-globulin fraction II was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). Triton X-100 was purchased from Rohm and Haas (Philadelphia, Pa.).

Results. When suspensions of the LGF were incubated in isotonic sucrose, β-glucuronidase activity was solubilized with time (Fig. 1). After 60 min, approximately 25% of the total LGF β-glucuronidase activity had become nonsedimentable. However, in the presence of cytosol only ca. 6% of the total LGF β-glucuronidase activity became solubilized within 60 min. Cytosol alone contained some β-glucuronidase activity which was stable to incubation at 37° and nonsedimentable.

Control experiments demonstrated that β-glucuronidase activity was not inhibited by cytosol and that the activity was stable to incubation at 37°. The LGF activity, solubilized by Triton X-100, for example, was stable during incubation in sucrose or in cytosol (Fig. 1A). In addition, the measured activity of the cytosol + Triton-solubilized LGF was the sum of the parts. Solubilization of β-glucuronidase from the LGF was dependent on the concentration of cytosol incubated with the LGF in the range of 3 to 16 mg of cytosol protein/ml of incubation mixture.

Dialyzed or boiled cytosol had the same effect as untreated cytosol on the solubiliza-

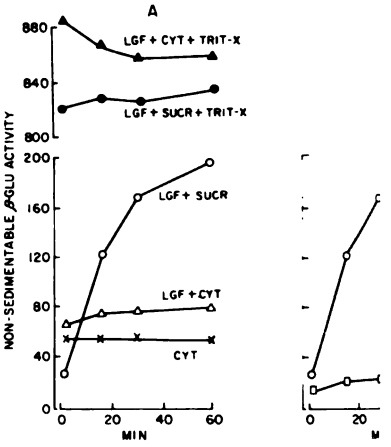

FIG. 1. Effect of sucrose or cytosol on t zation of β-glucuronidase of the large granu (LGF). The LGF (containing 22 mg of pr was suspended in either isotonic sucrose or mg of protein/ml) in a total volume of 7.5 m suspensions were incubated, with shaking, 7.2. Samples (0.5 ml) were either centrifug at 40,000*g* for 10 min or mixed with Triton subsequently centrifuged. β-Glucuronidase expressed in μg of phenolphthalein formed/ μl of sample. A. Nonsedimentable β-glu activity of the LGF incubated with sucrose cytosol (Δ) or of the cytosol alone (×) i isotonic sucrose; total activity of the LGF (●) or in cytosol (▲) was also determined. B A except that the activity in the cytosol alo been subtracted from the measured nonse LGF plus cytosol activity (Δ) to yield the ne of the mixture (□). This is a representative selected from 12 replications. For the LGI with sucrose, after 60 min, 22 ± 1% (mean error of the mean) of the total β-glucuronid had become nonsedimentable. In the prese sol, 6.0 ± 0.6% of the total LGF β-glu activity was nonsedimentable after 60 min tion.

tion of β-glucuronidase from the L 2). No significant differences were between the effects of boiled cytos quently dialyzed or dialyzed cytosol subsequently boiled. Boiling dest glucuronidase activity in the cytos as other enzymatic activities (e.g., and malic dehydrogenase). Boile did not affect the solubilization of a peroxisomal enzyme, or malic genase, a mitochondrial enzyme Experiments with acid deoxyribo and acid phosphatase (not shown),

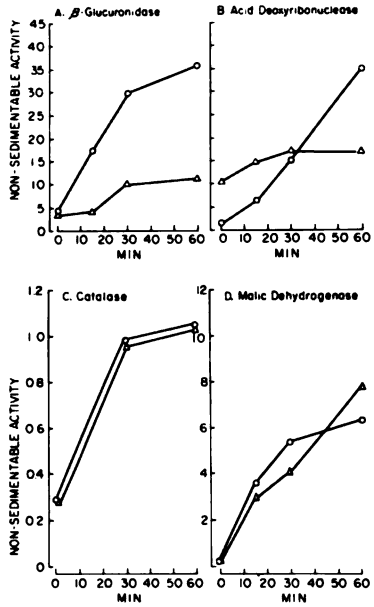

FIG. 2. Effect of boiled cytosol on the solubilization of enzymes from the LGF. The LGF was suspended in isotonic sucrose (O) or in boiled cytosol (Δ) (16 mg of protein/ml) and incubated at 37°. A. β-Glucuronidase is expressed in μg of phenolphthalein formed 30 min^{-1} 500 μl^{-1} of sample; B. Acid deoxyribonuclease is expressed in μg of deoxyribonucleotides formed 30 min^{-1} 500 μl^{-1} of sample; C. Units of catalase were k min^{-1} values (first-order rate constant) 50 μl^{-1} sample; and D. Units of malic dehydrogenase were μmole of β-NADH consumed min^{-1} 10 μl^{-1} sample. These are representative experiments. For β-glucuronidase, five additional experiments were conducted; for each of the three other enzymes, three additional experiments were conducted.

somal enzymes, indicated that solubilization of these enzymes was depressed by cytosol.

Cytosol added to the LGF 2 min prior to centrifugation was just as effective in causing β-glucuronidase activity to sediment as cytosol present continuously during the 37° incubation (Fig. 3). In contrast, cortisone acetate retarded the release of the enzyme if present during incubation but did not cause coprecipitation of β-glucuronidase when added to the LGF incubation mixture 2 min before centrifugation.

Soluble β-glucuronidase activity in the cytosol or derived from a hypotonic lysate of the LGF and then mixed with cytosol, sedimented in the presence, but not the absence, of the LGF membranes (Table I). Thus coprecipitation of β-glucuronidase activity was only observed when cytosol and LGF membranes were both present in the incubation mixture.

Albumin and γ-globulins also reduced the solubilization of β-glucuronidase whether added during incubation of the LGF or 2 min before centrifugation of the LGF preincubated in sucrose (Fig. 4). These serum proteins did not inhibit β-glucuronidase activity. In other experiments, RNA (25 mg/ml of isotonic sucrose) and heparin (25 mg/ml of isotonic sucrose), had no effect on the solubilization of β-glucuronidase at neutral pH. In the experiments described heretofore, the pH of the incubation mixture was pH 7.2. When the LGF was incubated at pH

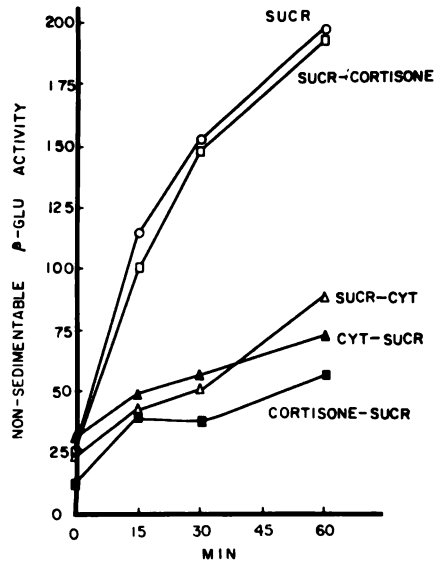

FIG. 3. Effects of cytosol and cortisone on the solubilization of β-glucuronidase activity. The LGF was suspended in isotonic sucrose (O), cytosol, 28 mg of protein/ml (▲), or cortisone acetate, 1.25 mg/ml of isotonic sucrose plus 1% dimethyl sulfoxide (DMSO) (■). Samples were removed from the cytosol (▲) and cortisone (■) suspensions, mixed with an equal volume of isotonic sucrose, incubated for an additional 2 min at 37°, and then centrifuged. Samples were also removed from the sucrose suspension at various intervals and mixed with an equal volume of isotonic sucrose (O), cytosol (Δ), or cortisone acetate in isotonic sucrose plus 1% DMSO (□); these samples were then incubated for an additional 2 min before centrifugation. The β-glucuronidase activity in the cytosol alone has been subtracted. DMSO (1%) alone did not affect the solubilization of β-glucuronidase. This is a representative experiment selected from three replications.

TABLE I. EFFECT OF THE HYPOTONIC SEDIMENT (MEMBRANES) OF THE LARGE GRANULAR FRACTION (LGF) ON SEDIMENTATION OF β-GLUCURONIDASE ACTIVITY[a].

Fractions	β-Glucuronidase activity		
	Prior to centrifugation	After centrifugation	
		Soluble	Sedimentable
Lysate	60	58	1
Membranes	41	29	10
Cytosol	48	41	6
Lysate and cytosol	100	97	5
Membranes and cytosol	88	32	53
Membranes and lysate	100	84	13
Membranes and lysate and cytosol	140	53	96

[a] The lysate (hypotonic lysate of the LGF) and membranes (hypotonic sediment of the LGF) were prepared as described in the materials and methods section and were diluted 20-fold prior to use. All tubes contained 1 ml of lysate, membranes, or cytosol, or mixtures of these solutions. The volumes of all mixtures were adjusted to 3.0 ml with 0.01 *M* Tris-HCl buffer, pH 7.2. The mixtures were incubated at 37° for 2 min. These experiments were repeated three times with different LGF preparations and the results were similar to those shown above.

5, cytosol or albumin did not sediment β-glucuronidase activity.

Discussion. In many studies on lysosomal stabilization *in vitro* it is assumed that the lysosomal membrane is stabilized when a biochemical agent appears to prevent the solubilization of a lysosomal enzyme and there is less leakage or release of the activity into the medium. On the basis of the results of the present work, this assumption warrants reexamination, for it is not justified under certain conditions. It has been demonstrated that β-glucuronidase cosediments with the LGF at pH 7 in the presence of cytosol, boiled cytosol, albumin, and γ-globulin. Thus, while these agents would *appear* to be lysosomal stabilizers, more likely they cause a coprecipitation of soluble lysosomal enzymes with cellular particles rather than stabilization of the membrane per se. These results are in contrast to those with cortisone acetate which truly is able to stabilize lysosomes.

The cosedimentation of lysosomal enzymes with the LGF in the presence of cytosol has some specificity, because a mitochondrial enzyme and a peroxisomal enzyme are not coprecipitated. Thus, cytosol may foster a selective interaction between the LGF membranes and specific enzymes.

The findings in I-cell disease (10), which indicate that lysosomal enzymes have a natural affinity for lysosomes, are pertinent to this work. Cytosol and serum proteins may be instrumental in promoting the interaction of lysosomal enzymes and lysosomes.

Other researchers have incubated protein solutions with lysosomes at pH 7. These protein solutions have been sera from (a) pregnant patients (11), (b) patients with chronic hepatitis and systemic lupus erythematosus (12), and (c) rabbits immunized against rat polymorphonuclear leukocyte membranes (13). These workers attribute

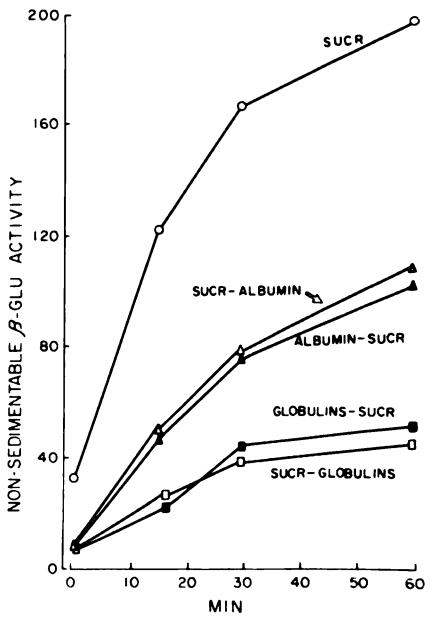

FIG. 4. Effect of albumin and γ-globulins on the solubilization of β-glucuronidase activity of the LGF. The LGF was suspended in either isotonic sucrose, an albumin solution, 23 mg/ml of isotonic sucrose (▲) or a γ-globulin solution, 22 mg/ml of isotonic sucrose (■). Samples were removed from the isotonic sucrose suspension, mixed with an equal volume of isotonic sucrose (○), the albumin solution (△), or the γ-globulin solution (□), and incubated for 2 min at 37°. Samples also were removed from the albumin suspension (▲) or the γ-globulin suspension (■), mixed with an equal volume of isotonic sucrose, and incubated for 2 min at 37°. These results were found consistently in four similar experiments.

the lower nonsedimentable lysosomal enzyme activity in the presence of certain serum samples to their stabilizing effects on the lysosomal membrane. Although test sera were compared to normal sera, it is quite possible that selective effects are due to cosedimentation of the lysosomal enzymes by components of the test sera.

The cosedimentation of lysosomal enzyme activity requires rather specific conditions; it occurs at pH 7 but not at pH 5 and the hypotonic sediment of the LGF must be present. The fact that boiled cytosol is able to affect cosedimentation of lysosomal enzymes indicates that subtle surface properties of proteins are not responsible for the interaction. On the other hand, the consistent finding that γ-globulins were more effective than albumin indicates that certain physical properties of the proteins in the LGF environment are determinative factors in cosedimentation. Important factors in this regard may be the net charge of a protein or the state of glycosylation of the protein. These observations raise the intriguing possibility that proteins having particular physicochemical properties may interact preferentially *in vivo* with lysosomes or other subcellular particles and that this may be determinative in the heterogeneity of half-lives of soluble intracellular enzymes.

Summary. Cytosol or serum proteins promote the cosedimentation of rat liver lysosomal enzymes with the large granular fraction membranes at neutral pH. Cytosol promoted cosedimentation of three lysosomal enzymes but not a mitochondrial or a peroxisomal enzyme. Dialyzed or boiled cytosol are as effective as untreated cytosol whereas γ-globulin is more effective than albumin in promoting cosedimentation.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 522-525 (1977)

Renal Response to Phosphaturic Agents in Acutely and Chronically Parathyroidectomized Rats (39708)

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Introduction. Extracellular volume expansion is associated with a decreased tubular reabsorption of phosphorus in both intact and chronically parathyroidectomized animals but not in acutely parathyroidectomized animals (1-6). The reason for this discrepancy is not entirely apparent, even though it was the subject of numerous investigations (4, 5). The present study was undertaken to extend the previous studies by examining the effects of several phosphaturic agents including diuretics in chronically and acutely parathyroidectomized rats with varying loads of filtered phosphorus. The diuretics were acetazolamide and chlorothiazide. Acetazolamide acts primarily on the proximal tubule to decrease fluid reabsorption in association with carbonic anhydraze inhibition and suppression of proximal bicarbonate reabsorption. Acetazolamide has the capacity to produce phosphaturia in both intact and chronically parathyroidectomized animals (7). Chlorothiazide suppresses sodium reabsorption, primarily in the cortical diluting segment of the distal nephron. Its effect on phosphate reabsorption is markedly smaller than that of acetazolamide.

Methods. White female Sprague-Dawley rats (180-300 g) fed Purina pellet chow diet with tap water *ad libitum* were studied.

Experimental procedures and groups. All rats were parathyroidectomized (PTX) by cautery either 2 hr (PTX − 2 hr) or 2 days (PTX − 2 days) prior to the start of clearance studies. These consisted of three to four 20-min control urine collection periods followed by five to six periods of the same length during which the drug was administered. Control rats and rats receiving the drug were always run concurrently. The surgical procedures and the chemical analyses were described previously (8-10).

The following groups were studied.

Group 1: administration of chlorothi (1.5 mg/100 g/hr iv); (a) PTX, 2 h PTX, 2 days; and (c) PTX, 2 hr plus sion of isotonic Na_2HPO_4 (6 mg/100

Group 2: infusion with 0.9% NaC ml/100 g/min); (a), (b), and (c) as in (1.

Group 3: infusion of acetazolamid mg/100 g/hr); (a) and (b) as in Group

Group 4: infusion of calcitonin (0.5 hr).

Results. All results are expressed as ± SE. The data presented in all table obtained by adding all 20-min urine c tions belonging to the same experin phases and calculating their arithmetic ages. The serum concentrations of pho rus and calcium were calculated in the fashion.

Chlorothiazide did not alter CP/C acutely PTX rats but it effected a signi increase in CP/CIN in chronically PT (Table I). Serum concentration and fi loads of phosphorus, before and duri infusion of chlorothiazide in group 1b higher than the corresponding valu group 1a, but the glomerular filtration did not differ significantly (Table II).

The administration of chlorothiazi acutely PTX rats receiving iv phosp effected a significant increase in Cl (Table I). There was no difference be the experimental and the control g during the infusion of phosphorus b increment in CP/CIN was noticed duri infusion of chlorothiazide resulting in crepancy of 0.071 between the group rum concentrations and filtered loa phosphorus in the control and the e mental groups did not differ significan

The effect of volume expansion 0.9% NaCl in acutely and chronically rats is shown in Table III. In group 2 ume expansion was associated with

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TABLE I. COMPARISON OF THE AVERAGE FRACTIONAL EXCRETION RATES OF PHOSPHORUS IN THE EXPERIMENTAL AND THE CORRESPONDING CONTROL ANIMALS, IN DIFFERENT GROUPS, DURING BASELINE AND EXPERIMENTAL PERIODS.

Group	N	CP/GFR Baseline clearances	CP/GFR Experimental clearances
a. EXP[a] Acute PTX + TZ[b]	6	0.010 ±0.002	0.012 ±0.002
CNT[c] Acute PTX	6	0.008 ±0.002	0.011 ±0.003
p[d]		NS	NS
b. EXP Chronic PTX + TZ	6	0.038 ±0.005	0.118 ±0.025
CNT Chronic PTX	6	0.030 ±0.009	0.038 ±0.001
p		NS	<0.01
c. EXP Acute PTX + P[e] + TZ	6	0.126 ±0.018	0.354 ±0.013
CNT Acute PTX + P	6	0.118 ±0.014	0.238 ±0.011
p		NS	<0.001
c. EXP Acute PTX + P + VE[f]	6	0.105 ±0.034	0.346 ±0.002
CNT Acute PTX + P	6	0.100 ±0.023	0.216 ±0.016
p		NS	<0.001
a. EXP Acute PTX + ACTZ[g]	6	0.002 ±0.001	0.159 ±0.010
CNT Acute PTX	6	0.002 ±0.001	0.003 ±0.001
p		NS	<0.001
a. EXP Acute PTX + CT[h]	6	0.0010 ±0.0002	0.044 ±0.018
CNT Acute PTX	6	0.0010 ±0.0004	0.002 ±0.001
p		NS	<0.01

[a] Experimental subgroup.
[b] Chlorothiazide was given during the experimental periods.
[c] Control subgroup.
[d] Compares the experimental with the control subgroups.
[e] Intravenous phosphorus was given during the baseline and experimental clearances in both subgroups.
[f] Extracellular volume expansion with saline was performed during experimental clearances.
[g] Acetazolamide was given during the experimental clearances.
[h] Calcitonin was given during the experimental clearances.

crease in urinary excretion of phosphorus and CP/CIN whereas in group 2a these variables did not change significantly. Similar results were reported in previous studies (4, 5). Glomerular filtration rates (CIN) and fractional excretion of sodium (CNa/CIN) in group 2a did not differ significantly from their respective values in group 2b, but serum concentrations and filtered loads of phosphorus in group 2b were higher than the corresponding values in group 2a (Table III).

The effect of volume expansion on renal handling of phosphorus in acutely PTX phosphate-loaded rats is shown in Table I. There was no difference in average CP/CIN between the experimental and the control animals during the infusion of phosphate. The addition of iv saline to the phosphate infusion in the experimental group produced an increment in CP/CIN, leading to a discrepancy between the experimental and the control group of 0.13. There was no significant difference in serum phosphorus concentrations, glomerular filtration rates, and filtered loads of phosphorus between the control and the experimental animals.

The effect of acetazolamide on renal handling of phosphorus in acutely PTX rats (group 3) is shown in Table I. There was no difference in the average CP/CIN between the experimental and control animals during the baseline collections. During the administration of acetazolamide to the experimental rats, average CP/CIN increased from 0.002 to 0.159, but in the control group CP/CIN did not change. In chronically PTX rats there was no difference in average CP/CIN between the experimental and control animals during the baseline collections. During the administration of acetazolamide to the experimental rats, average CP/CIN increased from 0.034 ± 0.003 to 0.19 ± 0.31 ($p < 0.01$), but in the control group CP/CIN did not change. Serum phosphorus concentrations, glomerular filtration rates, and filtered loads of phosphorus in the control and in the experimental animals did not differ significantly throughout the experiment, both in groups 3a and 3b.

The effect of calcitonin on renal handling of phosphorus in acutely PTX animals is shown in Table I. During the baseline, Cp/CIN in the experimental and the control animals did not differ significantly. During the infusion of calcitonin in the experimental rats CP/CIN increased from 0.001 to 0.044, but remained unchanged in the control group.

TABLE II. COMPARISON OF THE AVERAGE SERUM CONCENTRATIONS OF PHOSPHORUS, INULIN CLEARANCES, AND FILTERED LOADS OF PHOSPHORUS, BEFORE AND DURING CHLOROTHIAZIDE INFUSION IN ACUTELY PTX RATS WITH THE CORRESPONDING VALUES IN CHRONICALLY PTX RATS.

Group	Sp[a] (mg/100 ml)		GFR (ml/min)		Fp[b] (μg/min)	
	B[c]	TZ[d]	B	TZ	B	TZ
1a. Acutely PTX rats (N = 6)						
Mean	5.4	5.6	1.17	1.21	63	68
± SE	0.2	0.2	0.06	0.08	4	5
1b. Chronically PTX rats (N = 6)						
Mean	10.0	10.0	1.23	1.15	123	115
± SE	0.4	0.4	0.08	0.06	7	7
p[e]	<0.001	<0.001	NS	NS	<0.001	<0.001

[a] Serum phosphorus.
[b] Filtered load of phosphorus.
[c] Baseline clearances.
[d] Chlorothiazide was given during the experimental periods.
[e] Comparison of the acutely with the chronically PTX rats.

TABLE III. THE EFFECT OF EXTRACELLULAR VOLUME EXPANSION WITH NORMAL SALINE ON RENAL HANDLING OF PHOSPHORUS IN ACUTELY AND CHRONICALLY PTX RATS.

Group	GFR (ml/min)		Sp (mg/100 ml)		Fp (μg/min)		UpV[a] (μg/min)		CP/GFR		CNa/GFR	
	B[b]	VE[c]	B	VE	B	VE	B	VE	B	VE	B	VE
2a. Acutely PTX rats (N = 4)												
Mean	1.05	1.20	5.5	5.7	58	65	0.10	0.13	0.001	0.002	0.003	0.064
± SE	0.06	0.07	0.2	0.1	5	7	0.02	0.02	0.000	0.000	0.000	0.011
2b. Chronically PTX rats (N = 4)												
Mean	1.04	1.22	10.8	11.2	112	136	3.93	23.10	0.036	0.166	0.006	0.065
± SE	0.05	0.06	0.7	0.9	6	8	1.40	5.65	0.012	0.032	0.002	0.014
p[d]	NS	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS	NS

[a] Urinary excretion of phosphorus.
[b] Baseline clearances.
[c] Extracellular volume expansion was performed during the experimental clearances.
[d] Compares the acutely with the chronically PTX rats.

In chronically PTX animals during the baseline, CP/CIN in the experimental and the control animals did not differ significantly. During the infusion of calcitonin in the experimental rats CP/CIN increased from 0.040 ± 0.002 to 0.142 ± 0.031 (p < 0.01), but remained unchanged in the control group.

In all groups of rats in all experiments serum calcium concentrations were less than 6.5 mg/100 ml and did not change significantly during the course of the experiments.

Discussion. The present study demonstrated phosphaturic effects of iv calcitonin and acetazolamide in acutely PTX rats. The effect of calcitonin like that of PTH is mediated through the activation of the adenylate cyclase/cyclic AMP system in renal cortex (11, 12). Therefore, similarly to PTH, calcitonin is an effective phosphaturic agent in acutely PTX rats. There is no adequate explanation, however, for the similar effect of acetazolamide.

Both chlorothiazide and iv saline failed to enhance urinary excretion of phosphorus in acutely PTX rats but induced phosphaturia in chronically PTX rats and in acutely PTX rats undergoing iv loading with phosphate. The response to intravenous saline in acutely PTX rats after phosphate loading in this study confirmed previous results in dogs (5).

The failure of chlorothiazide and saline to increase urinary excretion of phosphorus in acutely PTX animals is not well understood, but two possible mechanisms are worth comment. First, because the bulk of filtered phosphorus in this situation is presumably

reabsorbed by PTH-responsive mechanism, and very little if any is reabsorbed by other mechanisms, suppression of PTH-independent systems for phosphorus reabsorption by chlorothiazide or saline loading cannot be an effective means of increasing urinary excretion of phosphorus. Second, although iv chlorothiazide and saline may suppress phosphorus reabsorption in acutely PTX rats it is eventually reabsorbed completely by the high capacity PTH-responsive system. The demonstration of phosphaturia with chlorothiazide and saline in phosphate-loaded acutely PTX rats implies that, as the reabsorptive capacity for phosphorus is deliberately exceeded, a greater proportion of filtered phosphorus is probably absorbed by PTH-independent mechanisms, that are readily suppressible by saline and chlorothiazide. The fact that the filtered loads of phosphorus in chronically PTX rats were higher than those in acutely PTX rats may at least partly account for the presence of the phosphaturic response to chlorothiazide and saline in chronically PTX rats, as opposed to its absence in acutely PTX animals.

Summary. Acutely PTX rats responded with phosphaturia to both iv calcitonin and acetazolamide but failed to respond to iv chlorothiazide or saline loading. Chronically PTX rats, and iv phosphate-loaded acutely PTX rats responded with phosphaturia to iv chlorothiazide and saline loading. These findings suggests that different agents may exert their phosphaturic effects by acting at different reabsorptive systems for phosphorus in the kidney.

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Received September 27, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 526-529 (1977)

Varying Effects of Deoxynucleosides on the Incorporation of Thymidine int of Monolayer and Suspension Cultures (39709)[1]

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Summary. Deoxyuridine will normally suppress the incorporation of subsequently added tritiated thymidine into the DNA thymine of cells grown in suspension culture to less than 10%. Conversely, deoxyuridine appears to stimulate thymidine incorporation, at times approaching thousandfold excess, when the cells are maintained as monolayers. This stimulation is time, temperature, and, to a lesser extent, pH dependent, but is not directly related to the density of the monolayer. Disruption of the monolayer reverts the cells to normal deoxyuridine suppression of thymidine incorporation into DNA. This study extends earlier evidence of variations in *de novo* and salvage DNA synthesis between suspension and monolayer cell cultures. We caution the equating of data obtained with suspension cell systems to solid tumor analysis.

Introduction. De novo DNA thymine synthesis has been monitored in short-term human bone marrow cultures by measuring the incorporation of tritiated thymidine ([^{3}H]TdR) into the acid-insoluble material (1). In normal tissues 0.1 μmole/ml of deoxyuridine (UdR) added prior to the addition of [^{3}H]TdR will effectively suppress the incorporation of [^{3}H]TdR into DNA to less than 10% (2). Lessening of the UdR suppression signifies defective *de novo* DNA synthesis and can be used as a sensitive indicator of abnormal folate metabolism (3). This UdR suppression test has been used to differentiate rapidly between folate and vitamin B_{12} deficiency in megaloblastic marrow (4) and also to evaluate the efficacy of the various folate antagonists such as methotrexate, pyrimethamine, and triamterene (5, 6). These data have since been extended to include drug human solid tumor tissue (7, 8 report the unexpected finding of tion of [^{3}H]TdR incorporation l bation with UdR or deoxycytidi monolayer cultures. We sugge utilization of deoxynucleosides significantly when monolayer r suspension cultures are used, anc to partially explain resistance to s of chemotherapy.

Methods and materials. Cell pr L1210 leukemia cells were har Hank's balanced salt solution (H taining heparin (500 U/ml) from aspirates of DBA/J2 mice 7 day ulation with 1×10^6 cells ip. The washed three times with HBSS the heparin and ascitic fluid pended in HBSS containing 33 fetal calf serum (FCS) to a conce $55\text{-}65 \times 10^6$ cells/ml. Cells iso human malignant ascitic and pl sions were treated as above. Hu marrow was aspirated from the and, after passage through a wi break up cell clumps and exclud was treated as described above. cells were grown in monolayers tissue culture plates (LUX) in M sential medium – Earle's salts 10% FCS and 4 m*M* glutamine. I lines were prepared by trypsir solid tumor specimens obtained and maintained through at least s as monolayer cultures in RPMI taining 10% FCS and 4 m*M* gluta cell lines derived from the solid tu not been carefully evaluated an represent malignant cells. Ch karyotypes were determined ess the method of Broder *et al.* (18). ber of chromosomes counted in th

[1] This research was supported by NIH Grants AM 16690-03, CA 14491-02A2, and The Chemotherapy Foundation of New York, Inc.

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eated cells ranged from 38–48. The ere determined to be of human origin presence of the marker chromosome umerous metacentrics. Isoenzyme is was used to exclude HeLa contami- Cell viability was determined by the blue dye exclusion test and all studies med with human tissues were done ing to the guidelines of the Declara- Helsinki. Informed consent was ob- for the collection of the bone marrow s.

rporation studies. 1–5 × 10^6 L1210 uman bone marrow, and cells from ant pleural and ascitic effusions preincubated with 0.1 μmol/ml of for 1 hr at room temperature. dR (1 μCi; ICN Pharmaceuticals; i/mmole) was added and the reactions llowed to proceed at 37° for 3 hr in a olic shaker (6). All reactions were med in triplicate and the total volume 0 ml of HBSS. Cells grown in mono- were plated into 16 × 75-mm tissue plates and used when the cells were ort of being confluent. The plates ashed three times with HBSS and the monolayer incubated as described . At the end of the incubation time the ere harvested by gentle scraping with er policeman and transferred to test or acid precipitation. In some experi- the cells were removed from the mon- before the start of the experiment eated in the same manner as the sus- n cultures.

l precipitation. The acid-insoluble material was precipitated with cold 10% trichloroacetic acid. Erythrocytes from the bone marrow cultures were removed by shock lysis prior to acid precipitation. The resultant material was dissolved in 1 ml of NCS reagent (Amersham/Searle) and counted in 15 ml of scintillation cocktail (toluene containing 30% ethanol, 0.6% PPO, and 0.3% dimethyl POPOP) in a Beckman LS 250 liquid scintillation system to a counting error of 2% or less.

Results. The presence of UdR significantly inhibits the incorporation of the [^{3}H]TdR in cells from malignant pleural and ascitic effusions, in the bone marrow system, L1210 cells, and established cell lines in suspension (Table I). In contrast to the suspension culture, there is a marked stimulation of [^{3}H]TdR incorporation by UdR in all monolayer cultures which in some instances reaches thousandfold increases (Table II). When the monolayers have been trypsinized and treated as suspension cultures, the [^{3}H]TdR incorporation is significantly suppressed by UdR (Table II). When the identical reactions were run at 4° there was no evidence of UdR stimulation of [^{3}H]TdR. The incorporation of [^{3}H]TdR into monolayer cultures is maximal at 15 min and remains unchanged over 2 hr, while the incorporation of [^{3}H]TdR in the presence of UdR is time dependent with maximal stimulatory effects not yet reached at 2 hr (Table III). The stimulatory effect of UdR on [^{3}H]TdR incorporation occurs in the HeLa cell monolayer whether they are confluent or in a growth phase of prolifera-

TABLE I. UdR SUPPRESSION OF [^{3}H]TdR INCORPORATION INTO THE DNA OF SUSPENSION CELLS.

Cell type	Diagnosis	[^{3}H]TdR incorporated in the presence of 0.1 μmole/ml of UdR (%)[a]
fluid (N.C.)	Mesothelioma	16.0
fluid (G.K.)	Mesothelioma	2.0
fluid (B.S.)	Adenocarcinoma, ovary	27.0
fluid (H.W.)	Adenocarcinoma, ovary	5.0
fluid (T.L.)	Adenocarcinoma, ovary	30.0
fluid (D.S.)	Adenocarcinoma, ovary	11.0
fluid (S.V.)	Adenocarcinoma, breast	20.0
arrow (average of 30 samples)	Normal	4.5
(average of 4 studies)		12.1
lymphocytes in culture (PGLC 33H)		3.0
leukemia cells (745A)		13.0

lue of 100% is [^{3}H]TdR incorporation in the absence of UdR.

TABLE II. EFFECT OF UdR ON THE [^{3}H]TdR INCORPORATION IN INTACT VS TRYPSINIZED MONOLAYERS.

Cell type	Condition	[^{3}H]TdR incorporated in the presence of 0.1 μmole/ml of UdR (%)
HeLa	a	439.0
	b	8.0
	b	14.0
Ca rectum (A.F.)	a	293.0
	a	379.0
	b	8.0
	b	38.0
Ca lung (T.M.)	a	>8000.0
	b	56.0
	b	39.0
Ca lung (R.R)	a	7266.0
	b	25.0
RD 114	a	360.0
	b	16.0
DBT II	a	445.0
	b	30.0
Normal lung (R.R)	a	169.0
	b	12.0
Ca lung (L.S.)	a	456.0
	b	12.0
Ca breast (Y.Y.)	a	151.0

a, intact monolayers.
b, trypsinized monolayer studied in suspension.

tion. Three-hour incubations in HeLa cell monolayers show that [^{3}H]TdR incorporation is pH dependent. In contrast, the [^{3}H]TdR incorporation in the presence of UdR is less pH dependent in the range of 7.27 to 8.32 (Table IV). [^{3}H]TdR incorporation in HeLa cell monolayers appears to increase with the degree of monolayer disruption while the reverse is true when UdR is present in the media (Table V).

BUdR (0.1 μmole/ml) blocks the stimulatory effect of 0.1 μmol/ml of UdR and, to a lesser extent, blocks [^{3}H]TdR incorporation in the absence of UdR in HeLa cell monolayer cultures. The stimulatory effect of UdR on the [^{3}H]TdR incorporation in the HeLa cell monolayer is still evident when the level of UdR ranges from 0.001 to 1.0 μmole/ml. This same phenomenon was observed when deoxycytidine (0.1 μmole/ml) was used instead of UdR. Uracil (0.1 μmole/ml) showed no inhibition or stimulation of [^{3}H]TdR incorporation while 0.1 μmole/ml of TdR suppressed the incorporation of the [^{3}H]TdR as expected.

Discussion. These studies measure different patterns of deoxynucleoside utilization for *de novo* DNA synthesis in monolayer and suspension cell cultures. Cells normally in suspension such as bone marrow or cell lines in suspension culture demonstrate UdR suppression of TdR incorporation into DNA. In contrast, cells grown in monolayer or those derived from solid tumors, whether loosely or densely populated, are apparently stimulated to incorporate more TdR when in the presence of deoxynucleosides such as UdR and deoxycytidine but not in the presence of uracil. This stimulation is time, temperature, and, to a lesser extent, pH dependent.

The intact HeLa monolayer incorporates less TdR per cell than when it is disrupted or in comparison to cell lines grown in suspension. Moreover, the monolayer cell lines reach an early plateau of TdR incorporation (30 min) as compared to cells in suspension (60–90 min). The reasons for these differences are not clear but suggest differences in the cell membrane transport system for deoxynucleosides. There is a distinct mem-

TABLE III. EFFECT OF TIME ON THE INCORPORATION OF [^{3}H]TdR IN MONOLAYERS (Ca LUNG).

Time (min)	[^{3}H]TdR (CPM)	[^{3}H]TdR in the presence of 0.1 μmole/ml of UdR (cpm)	Incorporated (%)
15	2770	1048	37.0
30	3039	2690	87.0
60	2292	4307	188.0
120	2384	6143	258.0

TABLE IV. EFFECTS OF pH ON THE INCORPORATION OF [^{3}H]TdR INTO THE DNA of HeLa CELL MONOLAYERS.

Start of incubation pH	[^{3}H]TdR (cpm)	[^{3}H]TdR in the presence of 0.1 μmole/ml of UdR (cpm)
7.27	9853	44346
7.25	9806	33655
7.50	12128	48453
7.90	18521	47195
8.32	19792	45743

LE V. EFFECT OF CELL DAMAGE ON THE [³H]TdR INCORPORATION INTO THE DNA OF HeLa CELL MONOLAYERS.

Status	[³H]TdR (cpm)	[³H]TdR in the presence of 0.1 μmole/ml of UdR (cpm)	Incorporated (%)
ıolayer	1843	13636	740.0
monolayer	19435	7880	48.0
monolayer removed from plate ıyed in test tube	22673	4337	21.0

:eration and structural change in the er cells when adapted to suspension (10) which may be concomitant :rations in the patterns of cellular sm. Myers and Feinendegen (9) cribed different patterns for combi- of deoxynucleoside utilization for nthesis depending on whether the e grown in suspension or on a glass Moreover, the different patterns the monolayer reverted to normal : monolayer was trypsinized and the pended. We have found similar ef- izing disrupted HeLa cell monolay- apparent, therefore, that the rela- s of incorporation of TdR and other cleosides are controled by a labile ınd that discrimination in favor of :ursor can only be defined for one ır set of conditions.

does not appear to be any single ting step in the incorporation of ex- nucleosides into DNA. TdR ap- enter the cell by active transport diffusion (12–14) and share a car- iated transport with UdR but not tidine (15). Phosphorylation occurs ıfter uptake (12, 13, 16) and these es are finally utilized for DNA syn- ıny of these steps could be crucial fferent conditions (14, 17).

JdR stimulation of [³H]TdR incor- may be due to a greater depend- the salvage pathway for DNA syn- monolayer cells and in cells derived lid tumors as compared with cells / in suspension. Reduction of TdR ım by UdR, a known competitive · of thymidine phosphorylase (11) use the monolayer cells (and solid ell) to incorporate greater amounts in the presence of UdR. BUdR n of the UdR stimulatory effect in er cells supports the concept that the reaction occurs prior to or during the phosphorylation of TdR. These observations reveal the necessity for extreme caution in correlating the data obtained from suspension cell studies to solid tumor analysis. Further studies are under way to determine the biochemical explanation for the difference in utilization of deoxynucleosides for *de novo* and salvage DNA thymine synthesis in monolayer and suspension cultures.

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Received September 22, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 530-533 (1977)

In Vitro and *in Vivo* Effects of Dimethylnitrosamine on Mouse Liver Mitoch Function[1] (39710)

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Summary. The *in vivo* and *in vitro* effects of the carcinogen dimethylnitrosamine (DMN) on the metabolic activity of mitochondria were studied. Oxygen consumption by mitochondria in state 3 and state 4 was determined polarographically following the addition of succinate or glutamate as substrates. The control of respiration by ADP is indicated by the ratio of state 3 to state 4 oxygen utilization (RCR), and is indicative of the tightness of coupling of the oxidative phosphorylation and electron transport systems. DMN in concentrations of 37.8 μM and higher significantly decreased state 3 oxidation of glutamate. State 4 oxidation of glutamate was inhibited significantly only at DMN concentrations of 75, 151, and 226 μM (5, 10, and 15 μg/ml). The RCR was decreased corresponding to the decrease in state 3 oxidation. Similar results, but to a lesser magnitude, were obtained using succinate as substrate. In mitochondria from mice treated with DMN (25 mg/kg ip) state 3 oxygen consumption was decreased and the RCR was significantly different from control with either substrate. The results indicate that DMN inhibits oxidative phosphorylation both *in vivo* and *in vitro*.

Introduction. Our laboratory has been involved in studies on the relationship between environmental compounds influencing mixed function oxidase activity and dimethylnitrosamine (DMN) metabolism. The basis for supposition of this interrelationship is the presence of dimethylnitrosamine demethylase activity in liver microsomes. Magee and Barnes (1) in their classic review on nitrosamines emphasized this relationship. More recently, Zeiger (2) reviewed the relationships among DMN demethylase activity, factors influer levels, and mutagenic activity of dim trosamine. Our laboratory, in stud tagonistic activity of alkylnitrosami methylenedioxybenzene derivatives methylnitrosamine, has generally su Zeiger's contentions (3). Howev clear toxicological parameter whic ures significance of this enzyme is n (LD_{50}) due to the ease of interpreta irreversibility of the response. Inhit murine DMN demethylase, however ally have not raised the DMN LD_{50} 20 mg/kg in mice (4). In order to this paradox, toxic effects of DM investigated. We report here that I an inhibitor of oxidative phosphoryl *vitro* and that DMN also inhibits o phosphorylation *in vivo*. The assay employed measured oxygen consum metabolic state 4[2] and state 3[3] of mi dria as defined by Chance and Willi

Methods and Materials. Male Swis mice were housed in shoe-box typ and fed Purina chow and water *ad* They were sacrificed by cervical disl and the livers were removed and h nized in ice-cold 0.25 *M* sucrose w 10^{-4} *M* EDTA. Mitochondria were by the method of Schneider and Ho (6, 7) and used immediately.

Oxygen consumption was meas larographically with a Clark fixed polarizing probe (7). Rate of oxyge tion was measured both in the pres absence of ADP, and is expressed moles of atomic oxygen (nanogra equivalents of oxygen) consumed p

[1] Supported by NIH Grant ES00701.

[2] State 4, resting state respiration (velc ADP exhaustion).

[3] State 3, active state respiration (rate of oxidation in presence of phosphate acceptor,

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ISSN

of mitochondrial protein per minute. espiratory control ratio (RCR)[4] is the f state 3 to state 4 respiratory veloci-). In addition to 2 mg of mitochon-rotein the reaction mixture contained *M* mannitol, 0.33 m*M* EDTA, 3.5 m*M* 3.5 m*M* potassium phosphate, and 2.4 bovine serum albumin, in a total vol-f 1.8 ml (7). All determinations were at 30°.

strates were titrated pH 7.3 and during incubation to concentrations m*M* succinate (pH 7.4) or 1.4 m*M* iate (pH 7.4), and 0.14 m*M* ADP (7). ie *in vitro* studies, DMN diluted in d water was added to the reaction er and preincubated with the mito-ria for 3 min before the determination tarted. Animals used in the *in vivo* s were injected with 25 mg/kg ip of , 30 min prior to sacrifice. The mito-ria were isolated and handled in the manner as those for the *in vitro* stud-ach experiment was repeated on at three separate occasions. Mitochon-protein was solubilized with 0.75 *M* I and determined by a biuret method.

ults. The results of incubating DMN iurine liver mitochondria are shown in . With glutamate as the substrate, the atory control ratio (RCR) was 3.82. ions of DMN induced a marked de-in the RCR. At concentrations of *μM* DMN the RCR was 85% of con-Similar suppression of state 3 oxygen mption took place with a 20% inhibi-: 37.8 *μM* and a 60% inhibition at 226 oncentration. State 4 oxidation was ised at the three higher doses by 29, nd 22% at 75, 151, and 226 *μM* , respectively. Each of these three is statistically different from control. cinate oxidation does not appear as ve as glutamate oxidation (see Fig. 2). ion of ADP to those mitochondria d an RCR of 2.63. Only at 75 and 226)MN were the RCRs statistically dif-from controls. At higher concentra-of DMN (75, 151, and 226 *μM*) the RCR was suppressed by 7, 9, and 29%, respectively. State 4 oxidation was slightly below controls throughout the experiment reaching inhibitory levels of 22% at the two highest concentrations. These were the only

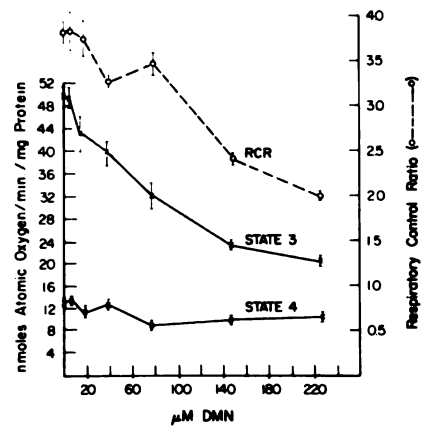

FIG. 1. *In vitro* effect of dimethylnitrosamine on mitochondrial oxidation of glutamate. Oxygen consumption was measured polarographically in the presence of ADP (state 3) and after exhaustion of ADP (state 4) using L-glutamate as substrate. Mitochondria were isolated from livers of male Swiss mice. Distilled water or DMN was added to the reaction mixture (total volume, 1.8 ml) and preincubated with the mitochondria for 3 min.

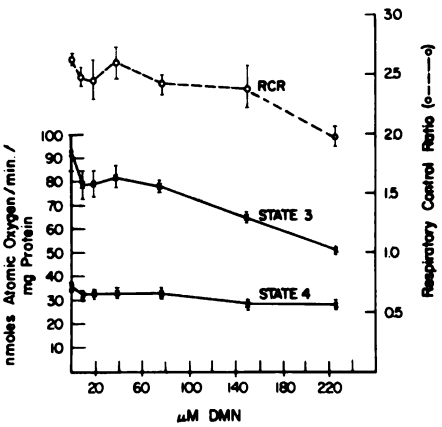

FIG. 2. *In vitro* effect of dimethylnitrosamine on mitochondrial oxidation of succinate. Oxygen consumption was measured polarographically in the presence of ADP (state 3) and after exhaustion of ADP (state 4) using succinate as substrate. Mitochondria were isolated from livers of male Swiss mice. Distilled water or DMN was added to the reaction mixture (total volume, 1.8 ml) and preincubated with the mitochondria for 3 min.

R, respiratory control ratio (ratio of active state ng state velocities), a measure of the control of ion by phosphate acceptor.

TABLE I. EFFECT OF *in Vivo* ADMINISTRATION OF DMN ON MITOCHONDRIAL RESPIRATION

Treatment	Number of mice	Substrate	RCR	Oxygen consumption, state 3	(Mean st
Control	4	Succinate	2.63 ± 0.07	92 ± 5	35.0
DMN	3	Succinate	1.97 ± 0.20*	80 ± 4	40.7
Control	6	Glutamate	3.45 ± 0.14	42.0 ± 3.8	12.1
DMN	5	Glutamate	2.57 ± 0.13*	32.9 ± 3.8**	12.8

[a] Male Swiss mice were treated with 25 mg/kg, i.p. DMN, and killed 30 minutes later. Mitoch isolated from the liver, and oxygen consumption measured polarographically.

[b] Exressed as nanomoles of atomic oxygen per milligram of mitochondrial protein per minute.

* Denotes statistically different from control ($p < 0.01$).

** Denotes statistically different from control ($p < 0.05$).

determinations which were statistically different from controls. At 7.5 and 18.9 μM DMN, state 3 oxidation was suppressed 18 and 15%, respectively. Since state 3 was always affected to a greater extent than state 4, the RCRs were also always below controls. However, as we stated earlier, only the high doses were statistically significant.

Oxygen consumption by mitochondria of mice treated with DMN is shown in Table I. In the case of succinate oxidation, although depression of the state 3 velocity was not significant, the RCR was decreased 24%. As was the case *in vitro* glutamate oxidation was more sensitive to DMN than was succinate. There was no change in state 4 oxidation while state 3 oxidation was inhibited 22%. Therefore, the RCR was suppressed by 25%.

Purified mitochondria were tested for DMN demethylase activity. At no time did mitochondrial preparations produce any measurable formaldehyde when incubated with DMN and several cofactors. Microsomal preparations, under similar conditions, produce significant and easily detectable amounts of formaldehyde.

Discussion. There has been a long history of observations leading to the conclusion that enzymatic activation of DMN was essential for its biological activity. As early as 1956, Dutton and Heath (8) followed the rapid production of $^{14}CO_2$ from [^{14}C]DMN and concluded that DMN had to be enzymatically activated. This conclusion was further emphasized in Magee and Barnes' (1) review on carcinogenic nitroso compounds. They pointed to the absence of mutatgenic activity in microorganisms lacking the ability to enzymatically activate DMN, and to the finding that T_4 phage was una exposure to a 25% solution of I Magee and Hultin (10) had sh DMN alkylates biological mate Lean and Vershuuren (11) showe tary protein deprivation affected t dence and DMN demethylase acti same manner. Heath (12) stated d that DMN must be metabolically to cause its toxic effects and that t tabolized molecule was inactive.

With this wealth of congruous tion, it was very surprising to fi variety of inhibitors of DMN de such as piperonyl butoxide, nitr ine, dibutylnitrosamine, and dieth mine had no suppressing effect on LD_{50} (4). Therefore, a different m of action was hypothesized for toxicity—one which, perhaps, di volve enzymatic activation. Dol Campbell (13) have shown that i of rat liver mitochondria with afl results in inhibition of electron Therefore, similar experiments we taken to determine whether DM aflatoxin in this sense. The *in* presented here show that DMN, p its unmetabolized form (there is be absolutely conclusive about this oxidative phosphorylation with glu the substrate and, to some extent, cinate. However, alternate metab ways in mitochondria not involvin dehyde production still remain a p

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Received June 21, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 534-537 (1977)

Hydrophobicity of Neuraminidase-Treated Rabbit Interferon (39711)

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Introduction. Heterogeneity of interferon molecules in biophysical, biochemical, and biological properties seems universal in all species (1). The heterogeneous property, although little understood, may be contributed to by carbohydrate moieties of the interferon molecule, the polypeptide structure, or both (2). Using bovine serum albumin-bound Sepharose 4B affinity chromatography, Huang *et al.* first demonstrated the hydrophobic nature of interferon and that there are degrees of differences in hydrophobicity among human, mouse, and rabbit interferons (3). Interferon induced from human fibroblast cells demonstrated the greatest hydrophobic property, whereas rabbit serum interferon showed the least, according to the degree of affinity for the column (3). The terminal carbohydrate moiety of rabbit interferon is believed to be sialic acid → galactose (4). The present report demonstrates that removal of the sialic acid group from rabbit interferon by neuraminidase will affect its binding property in a bovine albumin bound Sepharose 4B column.

Materials and Methods. Induction of interferon. Rabbit interferon was produced in primary rabbit kidney cells with poly(rI):poly(rC), cycloheximide, and actinomycin D as described (5) with the following modification. After the removal of antimetabolites, the cells were replenished with medium 199 (Gibco) free from serum to minimize the content of nonspecific glycoproteins presented in serum. The medium fluid was collected 3 hr later and was dialyzed at 4° against two changes of 1 liter of phosphate-buffered saline (PBS) in 24 hr. The dialyzed fluid contained about 8000 IU of interferon/0.1 ml as assayed by a semimicro assay method (6) using the NIH reference standard. Specific activity of this interferon was about 10^5 IU/mg protein.

Neuraminidase treatment of interferon. Before the enzyme treatment, interferon was dialyzed overnight in Na-acetate (0.15 *M* NaCl, 20 m*M* $CaCl_2$; pH 5 and then treated with 25 units of neur dase from *Vibrio cholera* (Schwartz/ Lot No. AZ 2199) at 37° for 2 hr. feron thus treated lost about 20% activity. A control interferon samp treated with 25 units of neurami which had been heat inactivated at 8(hr. The neuraminidase used contain than 1.0 IU/ml of proteolytic enzym tivity measured by the method of Alk *et al.* (7).

Affinity chromatography. CNBr-ac Sepharose 4B (Pharmacia, Lot No. was swollen in 0.1 m*M* HCl for at lea before use. Bovine serum albumin (' ington) was bound to beads according method of Huang *et al.* (3). More tha of the serum albumin was bound beads. The albumin-bound beads wer soaked with 100 ml of 0.1 m*M* et amine (pH 8.0) for 1-2 hr at room tem ture. The beads were washed with 30(0.1 *M* $NaHCO_3$ containing 0.5 *M* Na then alternately washed with three cy 150 ml of 0.1 *M* Na-acetate, pH 4.5 taining 1 *M* NaCl and 150 ml of 0.05 / phosphate buffer, pH 8.0, containin NaCl according to Huang *et al.* (3) beads were washed with 150 ml of 0 Na-phosphate buffer, pH 7.4, cont 0.15 *M* NaCl followed by 300 ml o and were then packed in a 0.9 × column at room temperature and f washed with 30 ml of PBS before use

Neuraminidase assay. Neuraminidas assayed by the method of Warren (8) bovine submaxillary mucin (Worthir as substrate and *N*-acetyl neuramini (Calbiochem) as standard.

Protein assay. The protein in the fra ated interferon samples was assayed l fluorescamine method (9). The Lowr method (10) was used to check the feron in medium 199 after dialysis for

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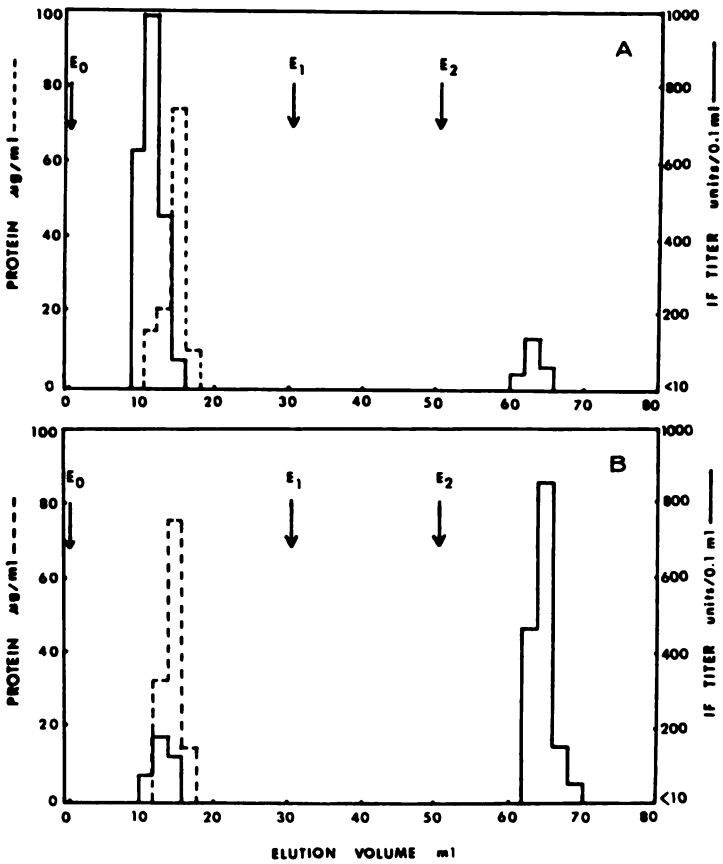
ino acids which may interfere with tein measurement by the fluorescaiethod. There was no significant inice of protein measurement by free acids in dialyzed interferon preparahen the fluorescamine method was

ts. Heterogeneity of rabbit inter-
A representative experiment shows elution pattern of the control interample is shown in Fig. 1A. The prontent in the interferon sample was by PBS (E_0) immediately after the lume. About 88% of interferon acould be found in the first several is eluted by E_0. The protein and in- peak of E_0 were too close together to be distinguished. There was no protein or interferon detected in a high salt eluent containing 0.02 *M* Na-phosphate and 1 *M* NaCl at pH 7.4 (E_1). The remaining 12% of the interferon activity bound to the column was eluted with 0.02 *M* Na-phosphate, pH 7.4, containing 33% by volume of a polar reducing agent, ethylene glycol (E_2). This elution pattern of interferon activity was not affected by the quantity of interferon applied to the column. These results indicate that poly (rI):poly (rC) superinduced interferon from kidney cells has two populations, one showing hydrophilic and the other showing hydrophobic properties.

The hydrophobic nature of neuraminidase-treated interferon. Figure 1B shows the



. Elution pattern of interferon. Two milliliters of interferon samples were layered on the column, by subsequent additions of 30 ml of E_0 (PBS), 20 ml of E_1 (0.02 *M* Na-phosphate, pH 7.4, containing 1 , and 30 ml of E_2 [0.02 *M* Na-phosphate, pH 7.4, containing 33% (v/v) ethylene glycol]. Collection of tarted immediately after the addition of samples. Approximately 2 ml of eluent were collected per (A) Interferon treated with heat-inactivated neuraminidase. (B) Interferon treated with neuraminidase. -----); interferon (——).

elution pattern of neuraminidase-treated interferon. After enzyme treatment, the total content of detectable protein was eluted by E_0 but only 20% of interferon activity could be found in the E_0 fractions. The majority of interferon activity (80%) had become hydrophobic and was found in fractions eluted by E_2.

Discussion. Removing sialic acid, a source of electrostatic force, from glycoproteins will increase the chances of other types of interaction of glycoprotein molecules, including a hydrophobic one. The experimental results suggest that the removal of sialic acid can affect the physical nature of interferon molecules. The finding that interferon treated with heat-inactivated enzyme showed a different affinity for albumin bound to Sepharose 4B beads indicated heterogeneity in the interferon population. After enzyme treatment, the majority of interferon molecules became hydrophobic. This change of affinity was not observed with rabbit serum interferon, probably due to the presence of abundant glycoproteins in serum which may interfere with the action of neuraminidase on interferon (data not shown). The minor peak eluted with E_0 in neuraminidase-treated interferon probably represented unaltered interferon. However, increasing the amount of enzyme to 50 units or the incubation time to 4 hr, or both, did not result in change in the elution pattern of interferon activity. The degree of neuraminidase action on interferon molecules is not known. The method we used to assay sialic acid was not sensitive enough to detect a measurable amount released from the interferon preparation. A control experiment using mucin as the substrate resulted in saturation of enzyme action under the conditions used for interferon treatment. Neuraminidase was eluted only by E_0 in a control experiment. The results suggest that the change of affinity of interferon to the albumin-bound Sepharose 4B beads after neuraminidase treatment and the difference in hydrophobicity among interferons are probably related to the content of sialic acid on interferon. Recently, Jankowski *et al.* (2) suggested that human leukocytic interferon is not a glycoprotein and is not hydrophobic,

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Received November 4, 1976, P.S.E.B.M. 1977, Vol. 154.

Lack of Caffeine Stimulation of (

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In 1944 Roth and Ivy (1) reported that caffeine given orally stimulates gastric secretion in man and the cat but not in the dog. They also reported that patients with duodenal ulcer had greater and longer responses to caffeine than did nonulcer subjects (2). With the introduction of histamine and other stimuli for gastric secretion, the caffeine test fell into disuse.

Recent physiologic studies by Debas and his colleagues have shown that the acid response to caffeine is linearly related to plasma caffeine levels (3). The earlier report of Roth and Ivy studying the combination of caffeine and histamine appears inconclusive (4). Furthermore, it has been shown that caffeine is not synergistic to pentagastrin stimulation (5).

Review of these data led us to inquire whether an aberration in gastrin release might be responsible for the apparently greater responsiveness of duodenal ulcer patients to caffeine and whether, in fact, the stimulation of gastric secretion by caffeine was mediated by the release of gastrin. Results indicated that it is not through gastrin release that caffeine stimulates gastric secretion.

Methods. In two patients with duodenal ulcer and in two normal controls, caffeine benzoate was given intravenously at the dose of 80 mg/hr for 2 hr and for 1 hr at 320

[1] Supported by NIH Gastroenterology Training Grant TIAM05286, Research Grant No. AM09260 from the National Institute for Arthritis, Metabolism and Digestive Diseases and the Medical Research Service of the Veterans Administration.

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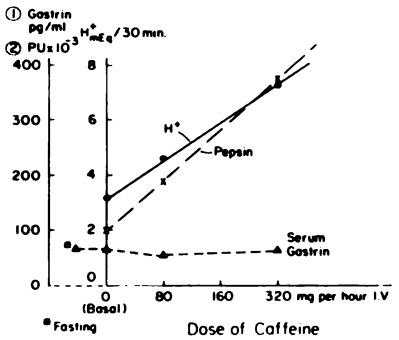
LE I. SERUM GASTRIN CONCENTRATIONS BEFORE AND AT INTERVALS AFTER 250 mg OF CAFFEINE IN 250 ml OF WATER IN SIX HEALTHY CONTROLS AND IN FIVE PATIENTS WITH DUODENAL ULCER (DU).[a]

	Mean (years)	Age (range)	Sex (M/F)	N	Fasting value	Mean change from fasting (pg/ml ± SE) 15 min	30 min	60 min	90 min
: control	34	25-63	4/2	6	83 ± 31	+11 ± 2.3*	+22 ± 6*	+14 ± 8	+33 ± 16
: DU	46	32-62	2/3	5	72 ± 8	+16 ± 6*	+12 ± 13	+5 ± 7	+23 ± 16
ontrol	38	32-44	0/3	3	31 ± 9	+1.3 ± 10	−1 ± 2	−11 ± 8	−9 ± 6

: equivalent volume of water in two of the DU and one of the control subjects produced no significant change in serum gastrin.
< 0.05.



. 1. Dose responsive (H^+) and pepsin output in ubjects (two normal, two DU). Basal secretion ollected for 60 min and serum gastrin was mea- at 0 and 60 min in the basal state. The 80-mg f caffeine was given for 2 hr and the 320-mg dose ven for 1 hr.

e. The increase in duodenal ulcer sub- was of the same order of magnitude. In our subjects showing a substantial rise stric H^+ and pepsin with caffeine given venously, there was no change in serum n. Cohen *et al.* (5) have shown that ne does not act synergistically with gastrin and also (3) that the degree of c response was proportional to the ntration of caffeine in the blood. It is unlikely that caffeine acts primarily by sing small quantities of gastrin and then ifying its effect through synergism as agus does. It is more likely that the is direct. The mechanism that imme- ly comes to mind is that of inhibition of hodiesterase, thereby increasing the ive cellular concentration of cAMP (7, ven this explanation must be ques- d in view of the nonresponse of dogs to ne (1). This has been confirmed in by Cooke *et al.* (9) and by us (unpub- l observation) by showing that neither ne nor aminophyllin directly stimu- nor acted synergistically with hista- or pentagastrin.

It remains to be shown by more direct means whether caffeine stimulates gastric secretion in man by a cAMP mechanism and, if so, whether it could act in the absence of another stimulus as in the amphibian (7, 8).

Summary and conclusions. Caffeine benzoate given intravenously to four subjects (two with duodenal ulcer and two normals) in dose-responsive tests at 80 and 320 mg/hr stimulated gastric H^+ and pepsin secretion two- and threefold, respectively, but did not change serum gastrin levels. In five patients with duodenal ulcer and six normals, 250 mg of caffeine benzoate intragastrically, but not an equivalent volume of water, raised serum gastrin concentrations by 16 to 22 pg/ml ($P < 0.05$). There was no difference in caffeine-stimulated gastrin release between the duodenal ulcer group and normals.

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Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 540-542 (1977)

Emetic and Hyperthermic Effects of Centrally Injected Methionine-Enkephalin in Cats (39713)

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Methionine- and leucine-enkephalins are endogenous pentapeptides which have been isolated from both pig (1) and bovine (2) brain and which have been shown to cause opiate-like effects such as analgesia (3-6) and physical dependence (7) in rats and mice. Morphine and related agents cause both emesis (8, 9) and hyperthermia (10) when injected into the cerebral ventricles of cats. This study was performed to determine whether methionine-enkephalin can also cause these effects in the cat and, if so, to learn if naloxone is an effective antagonist.

Materials and methods. Nine adult cats were prepared with third cerebral ventricular cannulas (11) and retroperitoneal thermocouples (12). All glassware was rendered nonpyrogenic by sterilization in dry heat at over 200° for at least 2 hr. Commercial, nonpyrogenic, disposable syringes and needles were used for injections. Environmental temperature was maintained at 22 ± 1°. The initial injection each day was given at 10:00 AM (±5 min), and the average of temperature readings recorded automatically at 9:30, 9:45, and 10:00 AM was used as the baseline from which changes were measured. Naloxone hydrochloride (Endo Laboratories) and morphine sulfate (Mallinckrodt) were dissolved in commercial, nonpyrogenic 0.9% saline solution shortly before use. Doses of these drugs refer to their salts. Methionine-enkephalin (Calbiochem) was prepared similarly except that it was necessary to acidify the saline to pH 3-4 with hydrochloric acid to dissolve enough of the enkephalin for injections of 1 and 2 mg. Saline solutions of similar acidity have been shown not to appreciably alter body temperature within 4-5 hr after injection (13). All agents were injected in a volume of 50 μl. The cannulas were flushed with 100 μl of saline solution after recovery from any hyperthermic response or on the day following a drug injection. In crossover studies of the effect of the enkephalin or morphine in animals pretreated in random order with and without naloxone, saline or naloxone was given at 10:00 AM and the enkephalin or morphine was given 30 min later. Enkephalin and morphine injections were spaced at least 72 hr apart to avoid possible development of tolerance. This interval between injections of morphine is sufficient to avoid tolerance in the cat (10).

Results. Doses of methionine-enkephalin of 0.5 mg or less did not cause emesis (Table I). However, 1-2 mg evoked vomiting on at least one occasion in all cats tested. In only 2 of 19 tests was emesis not evoked. In crossover studies with 2 mg of enkephalin, vomiting did not occur if the cats were pretreated with naloxone (100-250 μg). These doses of naloxone alone did not cause emesis.

A dose of 1-2 mg of methionine-enkephalin was likewise required to evoke shivering and definite elevations of body temperature (Table II). Responses began within 10 min and lasted approximately 2 hr, with the maximum rise occurring 30-45 min after injection. In one crossover study with intervals of 5-6 days between tests, 100 μg of naloxone reduced the maximum increase of body temperature in three of five cats. In a similar study with an interval of 12 days between tests, 250 μg of naloxone reduced

TABLE I. EMETIC RESPONSES TO THIRD VENTRICULAR INJECTIONS OF METHIONINE-ENKEPHALIN.

Dose (mg)	Number of cats	Responses/ tests	Latency (min)	
			Mean	Range
0.5	3	0/3	–	–
1.0	7	8/9	4	1-6
2.0	6	9/10 (0/10)[a]	5	3-8

[a] Naloxone (100-250 μg) injected into the third ventricle 30 min before enkephalin.

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ISSN 0037-9727

TABLE II. HYPERTHERMIC RESPONSES TO THIRD VENTRICULAR INJECTIONS OF METHIONINE-ENKEPHALIN AND MORPHINE SULFATE.

	Dose (mg)	Number of cats	Mean (range) maximum increase in body temperature (°)	
			Pretreatment	
			Saline	Naloxone
Methionine-enkephalin	0.5	3	0.4 (0.2-0.8)	–
	1.0	7	0.8 (0.3-1.1)	–
	2.0	5	1.2 (0.5-2.0)	0.8 (0.2-2.1)[a]
	2.0	5	1.2 (0.6-1.7)	0.7 (0.1-1.3)[b]
Morphine sulfate	10.0 μg	6	1.0 (0.3-2.0)	0.2 (0.0-0.9)[b]

[a] Naloxone (100 μg) 30 min before enkephalin.
[b] Naloxone (250 μg), $P < 0.05$ by paired t test.

the maximum response of all five cats. This latter dose of naloxone completely prevented the usual hyperthermic response to 10 μg of morphine sulfate in five of six cats (Table II). Neither naloxone alone nor the acidified saline vehicle appreciably altered body temperature. Other effects noted after enkephalin injections were mydriasis and vocalization.

Discussion. The above results demonstrate two central morphine-like actions of methionine-enkephalin in the cat. Inhibition of the responses by naloxone indicates that the enkephalin acted via opiate receptors. Naloxone has previously been reported to inhibit the emetic response to centrally administered morphine in the cat (9). The hyperthermic action of enkephalin seemed relatively more resistant to antagonism by naloxone than that of morphine, which was usually completely prevented by such pretreatment. Aside from emesis and hyperthermia, other effects elicited by morphine such as mydriasis and vocalization (14) were also seen after enkephalin administration. Based on the amount of agonist required to cause comparable increases in body temperature, 1–2 mg of methionine-enkephalin was about as potent as 10 μg of morphine sulfate. It has been estimated that at least 40 times as much enkephalin as morphine is required for comparable analgesic responses in the rat (6). The duration of hyperthermic action of the enkephalin was much shorter than that of 10 μg of morphine in the cat (10). The low potency and short duration of action of enkephalin was probably the result of rapid enzymatic degradation (6). It is unlikely that endogenous enkephalins function normally to control body temperature since pure opiate antagonists have been shown not to markedly alter body temperature in nondependent animals, even under conditions of thermal stress (15).

Summary. Third cerebral ventricular administration of 1–2 mg of methionine-enkephalin caused both emesis and hyperthermia in cats. The emetic response was prevented, and the hyperthermic response was reduced by 100–250 μg of naloxone given intraventricularly 30 min before enkephalin. These results provide further evidence that enkephalins can act on opiate receptors in the brain.

This investigation was supported by USPHS Research Grant NS 08618. I would like to thank Ms. Jean Ann Robins for her technical assistance.

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Received December 28, 1976. P.S.E.B.M. 1977, Vol. 154.

OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 543-545 (1977)

ıd Plasma Glucocorticoid Alterations After Injections of Hydrocortisone and Adrenocorticotropin (39714)

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ılogical relationships between blood k concentrations of estrogens and ;rone have been shown during the ;strous cycle and during pregnancy 3lood concentrations have been re- o be of lower magnitude than milk ·ations. Glucocorticoid concentra- milk during lactation (3) and after orticotropin (ACTH) injection (4) ;n reported in two recent abstracts. r, the relationships between blood ; glucocorticoid concentrations were uated. In several clinical conditions, glucocorticoids, synthetic glucocor- and ACTH are administered thera- y. The concentrations of glucocorti- milk and plasma after treatment ·enal product substitutions or adre- ulating compounds should therefore mined.

bjective of this study was to deter- ınges in plasma and milk glucocorti- ıcentrations after intramuscular in- ɔf hydrocortisone and ACTH.

ials and methods. Eighteen Holstein ree from intramammary mastitis ns were used. Ten cows, producing ıge of 16 kg of milk/day, were in- ıtramuscularly with 1600 mg of Δ^4- -11β,17α,21-triol-3,20-dione (hy- sone; Sigma Chemical Co., St. Mo.) after the 0800-hr milking. ıws, producing an average of 18 kg day, were injected im immediately l 7 hr after the 0800-hr milking with 50 (n = 6) or 100 (n = 2) IU of (porcine ACTH, Sigma Chemical Louis, Mo.).

ıilk samples (80 ml) were collected lly from each mammary quarter be- 0800-hr milking and 15 hr later. ry quarter samples were pooled im- ly, and a 40-ml aliquot was poured designated as the whole milk fraction. The rest of the sample (280 ml) was centrifuged at 5000g for 30 min at 5°, and the skim milk fraction was removed. Milk fractions were frozen and stored at −20°.

Immediately after collection of the foremilk samples, tail vein blood samples were collected via vena puncture in heparinized syringes. Blood was placed immediately into an ice bath and later centrifuged at 5000g for 30 min at 5°. The plasma was harvested and stored at −20°.

Total glucocorticoids in milk and plasma were quantified using the competitive protein binding assay (5) as modified by Gwazdauskas *et al.* (6). During the analysis of glucocorticoids in milk, approximately 1500 cpm of radioactive cortisol [1,2-^{3}H(N)]hydrocortisone (New England Nuclear) was added to each 20-ml glass culture tube as an internal standard to correct for procedural losses. Samples of milk (1-ml or 0.5-ml) were extracted twice, each with five volumes of iso-octane. These extracts were discarded. The rest of the milk residue was then extracted by vigorous shaking for 1 min with methylene chloride. After freezing, the organic residue was poured into a test tube and evaporated to dryness in a water bath (40°) under air. The sample was then reconstituted with 2 ml of methylene chloride:methanol (9:1). A 0.2-ml aliquot was taken to estimate recovery. Aliquots of 0.1 and 0.2 ml or 0.3 and 0.6 ml were dried and assayed for total glucocorticoids (6). The duplicate estimates on each sample satisfied the dose-response relationships. Validity of this system for determination of milk glucocorticoids was shown by the addition of known amounts of nonradioactive cortisol to aliquots of milk and extracting and assaying as described (Table I).

Statistical evaluations of factors influencing final glucocorticoid concentrations in plasma, whole milk, and skim milk were by

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ISSN 0037-9727

least-squares procedures. Each model included effects of treatments and covariates on preskim milk, prewhole milk, and preplasma concentrations of glucocorticoids.

Results and discussion. Results in Table II show the means and standard errors for glucocorticoids in plasma, whole milk, and skim milk before and after hydrocortisone or ACTH injections. Injecting 1600 mg of hydrocortisone resulted in 2.7-, 8.0-, and 5.0-fold increases in glucocorticoids in plasma, whole milk, and skim milk, respectively. Paape *et al.* (7) infused hydrocortisone (1600 mg) into the mammary gland of lactating dairy cows and reported plasma glucocorticoid concentrations of 127 ng/ml 3 hr later. The concentration of glucocorticoid in whole milk from the infused mammary quarters was over 400 ng/ml at 4 to 9 hr after infusion. The concentration in mammary quarters not infused was only 14 ng/ml at 4 hr after infusion, but basal concentrations were not reached until 48 hr

TABLE I. PRECISION OF GLUCOCORTICOID DETERMINATIONS IN BOVINE WHOLE AND SKIM MILK USING COMPETITIVE PROTEIN-BINDING QUANTIFICATION.

Cortisol (ng)			
Added	Measured	N[a]	SE[b]
Whole milk			
0	1.4	4	0.07
5	5.7	4	0.51
10	9.5	4	0.29
20	20.7	4	1.11
50	51.5	4	1.89
Skim milk			
0	1.2	4	0.22
5	4.9	4	0.52
10	12.5	4	1.48
20	19.9	4	1.26
50	58.1	4	1.76

[a] Number of samples assayed.
[b] Standard error.

TABLE II. GLUCOCORTICOID CONCENTRATION IN BOVIN AFTER HYDROCORTISONE

Treatment and dose	Number of cows	Plasma	
		Before injection	After injection
Hydrocortisone, 1600 mg	10	12.5 ± 2.4	34.3 ± 6
ACTH, 250 IU	6	9.5 ± 2.3	70.6 ± 1
ACTH, 100 IU	2	20.9 ± 6.9	109.1 ± 1

[a] Expressed in nanograms per milliliter ± SE.

TABLE III. CORRELATIONS OF GLUCOCORTICOIDS IN PLASMA, WHOLE, AND SKIM MILK.

	Whole milk before injection	Plasma before injection	Skim milk after injection	Whole milk after injection	Plasma after injection
Skim milk before injection	0.89[a]	0.54	0.53	0.44	0.56
Whole milk before injection		0.36	0.61	0.58	0.54
Plasma before injection			−0.04	−0.17	0.49
Skim milk after injection				0.95	0.68
Whole milk after injection					0.57

[a] $|r| > 0.45$ differs from 0 ($P < 0.05$).

Also, the final fluid concentrations of glucocorticoids were correlated to preinjection concentrations. These results suggest that glucocorticoid concentrations in mammary secretions reflect blood concentrations.

Although the pattern of milk glucocorticoids followed that of plasma glucocorticoids, the concentration of this steroid in both fluids was the reverse of that found for progesterone and estrogens (1, 2); there, progesterone and estrogen were found in higher concentrations in milk than in plasma. Our data suggest that although the mammary gland may actively take up and/or synthesize progesterone (2) and estrogens (1), it does not function the same with glucocorticoids.

The similarity of glucocorticoid concentrations in skim milk and whole milk suggests that glucocorticoids are not associated with milk fat, as has been shown with progesterone (2).

Summary. Intramuscular injections of hydrocortisone (1600 mg) or ACTH (250 or 100 IU) elevated the concentration of glucocorticoids in plasma and milk. The concentration of glucocorticoids in plasma was highly correlated with the concentration in whole milk and skim milk, both before and after either hydrocortisone or ACTH injections. The similarity of glucocorticoid concentrations in whole milk and skim milk fractions suggests that glucocorticoids are not primarily associated with milk fat.

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Received September 27, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 546-549 (1977)

Detection and Distribution of Intestinal Calcium-Binding Protein in the Youı Male Guinea Pig[1] (39715)

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Intestinal calcium-binding protein (CaBP) has been detected in a wide variety of animals including frog, carp, and pike (1, 2), chick (1, 3-5), rat (5-9), hamster (10), guinea pig[2] (13), dog (14), pig (1, 5, 13), bovine (15), horse (13), squirrel and cebus monkeys (16), and human (17-20). In at least the chick (3, 4), rat (6-8), dog (14), and monkey (16) CaBP is vitamin D-dependent, i.e., in the absence of vitamin D, synthesis of CaBP is reduced or absent.

Previously we attempted to produce rickets in the guinea pig using a Ca/P ratio of 1.1 to 1 and depriving the animals of exogenous vitamin D and light of wavelengths < 350 m, thereby excluding those wavelengths found to be antirachitic in rats (11, 12, 21). No evidence of rickets was found based on changes in plasma calcium concentration or by histological examination of the parathyroids and of the growth plate of the humerus. Since this attempt was unsuccessful and it was not yet known whether the guinea pig had intestinal CaBP, it was of interest to resolve the question.

Methods. Four male English s guinea pigs,[5] raised on commerci prior to sacrifice and weighing 300 were killed by exsanguination. Ren extraction of duodenal musoca we tially the same as described by W and Taylor (3) with slight modifica cosae from the four animals were p

Five additional guinea pigs from colony were used to compare the binding activity in different segmei intestine. The mucosae from the were pooled as follows: for the du the first 18 cm of the small intestin jejunum, the middle 18-25 cm of intestine; for the ileum, the last 18 small intestine; for the cecum, the 1/2; for the colon, all. The pooled were processed as described above supernatant was used for analysi cium-binding activity for each segn

The mucosal supernatant was ated on a 2.5 × 85-cm Sephade column which was eluted with pH buffer at 4°. The flow rate was 0.3 t min, and 2-ml fractions were collec fractions were analyzed for protein for calcium-binding activity by the 100 method (3). The Chelex-100 depends on the competition betwe ion-exchange resin and a calcium substance for added radiocalcium presence of a chelator or soluble coı factor, less ^{45}Ca is sequestered by t

To see whether the calcium-bindi was a charged particle and to visuali proteins in the mucosal supernat separated by gel electrophoresis ((23). Samples of 100 μl from fract 32, 46, 64, and 72 from the Seph filtration experiment (see Fig. 1) crude supernatant were run at pH 1.5 hr and then stained with buffal

[1] Supported in part by NIH Nutrition Grant GM01221, NIH Grant AM04652, and U.S.A.E.C. Contract E(11-1)-3167.

[2] The work reported here was initially presented at ASAS/ADSA Meeting, University of New Hampshire, July 16-18, 1972 (11, 12). It represents a portion of a thesis submitted by M. W. Chapman to the Graduate School of Cornell University in partial fulfillment of the requirements for the M.S. degree.

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[5] From a breeding colony which had been closed to the introduction of animals for 25 years; originally from a cross between animals of strain 13 from NIH and animals from Carworth Farms, New City, N. Y.

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To check further whether the calcium-binding factor was indeed a protein, it was subjected to enzymatic digestion by trypsin and by Pronase in a concentration of 100 μg of enzyme/ml of supernatant. The samples were incubated at 37° with shaking and aliquots of 0.1 ml were removed at 0, 15, 30, 60, 120, and 180 min and analyzed for calcium-binding activity as above.

Results and discussion. The elution diagram of Fig. 1 shows that the calcium-binding activity was associated primarily with the third major protein peak in the region of relatively low molecular weight substances and was associated with only a single major peak in the region of fraction 48. A much lower binding activity was associated with the column void volume and probably could be attributed to high molecular weight proteins and aggregates bearing negatively charged groups. The major calcium-binding peak was well-defined, and rose 60-fold above background. A similarly prominent peak in the elution pattern was reported for the chick (23), rat (6), bovine (24), and horse (13). If this peak is a single protein, the fact would be consistent with data for those CaBPs.

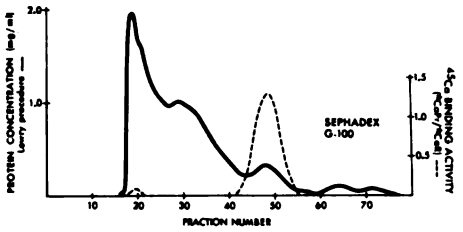

FIG. 1. Distribution of proteins and calcium-binding activity in fractions of supernatant after separation by dextran gel filtration (Sephadex G-100). Elution was performed with pH 7.4 Tris buffer at 4°, and 2-ml fractions were collected. Protein by the Lowry *et al.* procedure (22) and calcium-binding activity by the Chelex method (see text) were determined in aliquots of alternate fractions. (- - -) ^{45}Ca-binding activity expressed as the ratio of ^{45}Ca associated with the protein to that associated with resin (*CaPr/*CaR) (26); (——) protein concentration.

Figure 2 shows acrylamide gel electrophoretic patterns of several fractions of the Sephadex gel filtration and of the crude supernatant. The arrow indicates a prominent protein band in fraction 46 which may be the calcium-binding factor since this fraction corresponds to the calcium-binding peak seen in Fig. 1. Figure 3 shows the changes in calcium-binding activity with time for the incubation of 100 μg of trypsin or Pronase/ml of guinea pig intestinal supernatant. Pronase acted very quickly to destroy almost completely the calcium-binding activity, but trypsin had practically no effect. At the end of the incubation period (180 min) 80% of the original calcium-binding activity remained in the trypsin digest whereas only 6% remained in the Pronase digest. This is similar to what was seen in the bovine (15, 24) and in the dog (14). As previously pointed out (24), potential reasons for lack

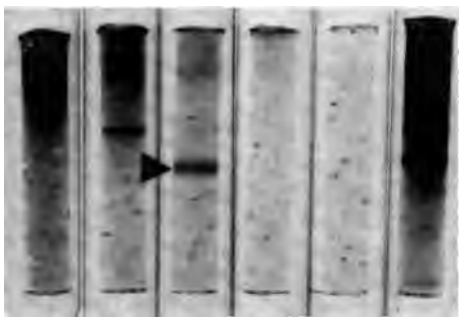

FIG. 2. Acrylamide gel electrophoresis pattern of protein in selected fractions of mucosal supernatant before and after gel filtration. Mucosae were pooled from normal pellet-fed guinea pigs. Arrow indicates probable CaBP band. Migration was toward the anode (bottom).

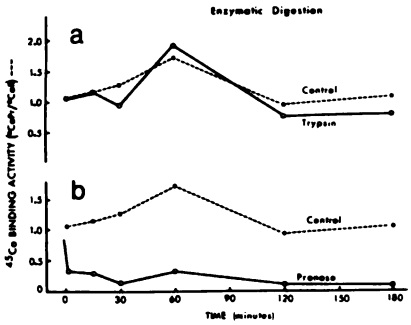

FIG. 3. Effect of trypsin and Pronase digestion on the calcium-binding activity of guinea pig CaBP compared to control. Incubation was at 37° for designated times and at enzyme concentration of 100 μg/ml of mucosal supernatant. (·- - - -·) intestinal supernatant with no enzyme added; (O——O) CaBP incubated with either trypsin or Pronase.

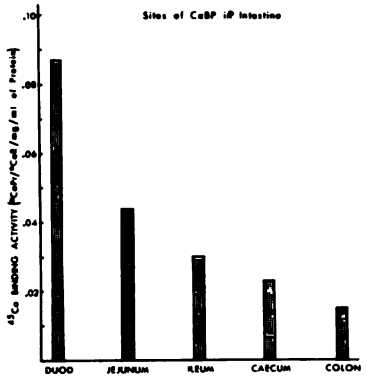

FIG. 4. Comparison of guinea pig CaBP activity as a function of intestinal site.

of digestion by trypsin are several including (a) lack of cleavage, since trypsin is quite specific and (b) cleavage at points too far removed from the active binding site(s) of the molecule to impair calcium-binding activity. However, it is now evident that trypsin-resistance is a function of the calcium content of the protein and the conformational masking of trypsin-susceptible groups on CaBP which might take place when the protein binds to calcium (25). The fact that Pronase so thoroughly digested the binding factor strongly suggests that it is a protein; in addition, what may have been the same factor in a fraction near peak ^{45}Ca-binding activity was charged and moved in an electric field.

Figure 4 shows that the guinea pig has calcium-binding activity throughout the GI tract with the activity decreasing with increasing distance from the stomach. This distribution is similar to that of the chick (23), squirrel and cebus monkeys (16), and the golden hamster (10). In the chick and hamster the rate of calcium absorption per unit segment correlates well with the relative amount of CaBP in a given segment (3, 10).

Summary. A calcium-binding factor was detected in guinea pig intestinal mucosa. It was negatively charged and moved in an electrical field. Pronase quickly destroyed almost completely the calcium-binding activity although trypsin had practically no effect. The major calcium-binding peak was well defined. It was concluded that the factor is probably a protein. Calcium-binding protein was found throughout the gastrointestinal tract, with the calcium-binding activity decreasing with increasing distance from the stomach.

The authors thank Dr. Walter L. Nelson for supplying the guinea pigs and Mr. Francis Davis and Mrs. Marie Brindak for their excellent technical assistance.

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Received July 22, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 550-552 (1977)

Failure of Bran to Alter Diet-Induced Hyperlipidemia in the Rat (39

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Epidemiological studies have shown that serum cholesterol is low in Africans who live south of the Sahara and consume large amounts of fiber in the form of natural carbohydrates and maize flour (1, 2). Dietary fiber is defined as the skeletal remains of plant cells which are resistant to digestion by luminal and brush-border digestive enzymes. Bran is the fiber-rich fraction of wheat which is eliminated during the milling of white flour. Bran has been increasingly used in the diet in recent years as a source of dietary fiber. Although bran exerts its effect in the colon, it has been recognized that it may inhibit the utilization of energy derived from the diet through a negative effect on fat and nitrogen metabolism (3). It has been proposed that plasma lipids and ischemic heart disease are related to a deficiency in vegetable dietary fiber (4, 5). In order to determine the influence of fiber on lipids, we have investigated the effect of dietary fiber in the form of bran on diet-induced hyperlipidemia in the rat, under controlled experimental conditions, in a long-term prospective study.

Methods. Sprague-Dawley male rats weighing 300-360 g were housed in individual cages. Feed and water were supplied *ad libitum*. Four groups of animals were studied for a period of 16 weeks. The composition of isocaloric diet (Teklad Test Diets, Madison, Wis.) in the different groups is shown in Table I. Group I was fed with a control diet with cornstarch; group II was fed with cornstarch and wheat bran (10%); group III was given an atherogenic diet with sucrose; and group IV was given an atherogenic diet with sucrose and wheat bran (10%). In order to determine t characteristics of the bran, test : used. The bran was shaken for the weight of the flour passed t mesh was recorded; 38% filtere 1.0-mm aperture size, 28% filte 0.5 mm, and 34% filtered thr mm.

Animal weight was recorded intervals and feed intake wa daily. At the end of a 16-week were sacrificed by decapitation was collected from the inferior Liver, heart, and aorta were re wet weight was recorded. Tissu liver, heart, small intestine, aort nary arteries was fixed in 10% hyde and stained with hemato for light microscopy.

Serum cholesterol was determ method of Wybenga *et al.* (6). glycerides were determined in isopropyl ether extract by the Azarnoff (7). Cholesterol and concentrations were determined extract homogenate with isopro according to the methods descri

Statistical significance of the d termined by the Student's *t* tes data.

Results. All groups of rats gai by 34-38%, as compared to weight, by the end of a 16-wee riod (Table II). There was no significant difference in the weig any of the groups. Animals w given bran had an increase in s and the consistency of the stool as compared to the dry pellet-li the animals which did not receiv

The results of serum and liver are summarized in Table II. Br effect on serum cholesterol and t in the control (cornstarch diet) a

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TABLE I. COMPOSITION OF DIET IN THE FOUR GROUPS OF RATS

Dietary components	Group I (%)	Group II (%)	Group III (%)	Group IV (%)
Casein, high protein	22.0	19.9	20.6	18.7
Cornstarch	62.3	56.0	–	–
Sucrose	–	–	58.2	52.8
Corn oil	9.0	8.1	–	–
Coconut oil	–	–	8.4	7.4
Mineral mix (Rogers–Harper)	5.5	5.0	5.2	4.6
Vitamin mix (Teklad)	1.1	1.0	1.1	0.9
Cholesterol	–	–	5.0	4.2
Cholic acid	–	–	1.5	1.4
Bran	–	10.0	–	10.0

TABLE II. EFFECT OF VARIOUS DIETS ON ANIMAL WEIGHT, SERUM, AND LIVER LIPIDS.[a]

Diet[b]	Initial weight (g)	Final weight (g)	Feed intake (g)	Serum cholesterol (mg/100 ml)	Serum triglyceride (mg/100 ml)	Liver cholesterol (mg/g body wt)	Liver triglyceride (mg/g body wt)
I. Control diet with cornstarch (19)	343 ± 6	458 ± 10	19 ± 2	186 ± 14	35 ± 5	6 ± 0.2	14 ± 1.0
II. Cornstarch with 10% bran (16)	349 ± 5	443 ± 15	20 ± 3	188 ± 14	33 ± 4	5 ± 0.1	13 ± 2.0
III. Atherogenic diet with sucrose (17)	352 ± 5	439 ± 6	22 ± 2	491 ± 62	65 ± 5	58 ± 9.0	20 ± 0.3
IV. Atherogenic diet with sucrose and bran (20)	348 ± 6	449 ± 9	21 ± 3	577 ± 50	94 ± 8	59 ± 7.0	18 ± 1.4

[a] Mean ± SE.
[b] Number of animals is given in parentheses.

perimental groups (atherogenic diet with sucrose). The difference in serum cholesterol between the control (group I) and the experimental (group III), as well as with group IV (atherogenic diet with bran), was highly significant ($p < 0.001$). No significant difference was found between group III and group IV.

Similarly, serum triglyceride levels were comparable in groups I and II, as well as in the experimental groups III and IV. There was a significant difference between group I and group III ($p < 0.005$), as well as between groups I and IV ($p < 0.05$). Bran added to the atherogenic diet not only failed to prevent hypertriglyceridemia, but resulted in a modest elevation of serum triglyceride.

Liver cholesterol and triglyceride levels were significantly higher in the experimental groups III and IV than in the control group I ($p < 0.001$). Bran did not influence the deposition of lipids to the liver in the cholesterol-fed animals.

Morphological changes. There was a marked degree of fatty metamorphosis of the liver in the group of rats given the atherogenic diet. The addition of 10% wheat bran in the diet did not prevent fatty liver. Liver weight in group III was 29 ± 1 g (mean ± SE) vs 11 ± 0.5 g in group I ($p < 0.005$). Liver weight in group III was comparable to that in group IV (29 ± 1 vs 27 ± 0.7). Heart and aorta weights were comparable in the four groups. There was no evidence of atherosclerotic plaque formation in the aorta in the groups given a high cholesterol diet. The heart, aorta, small intestine, and liver were normal by light microscopy in groups I and II. There was marked fatty metamorphosis of the liver in groups III and IV, but no changes were noted in the heart and aorta.

Discussion. The results of this study indicate that the addition of 10% bran in the diet in the rat did not exert any effect on hyperlipidemia induced by a high cholesterol diet. Similarly there was no effect on liver lipids or the fatty changes which occurred in the rats treated with a high cholesterol diet. It was also of interest that bran did not lower serum lipids in the control group of rats on a cornstarch diet. We elected to add bran in the diet in order to provide the animals with a more physiological dietary pattern, rather than administer

the bran in one dose. The addition of bran markedly influenced the colonic function of the rats in our study by increasing stool bulk and altering the consistency of stool. However, alteration in colonic function had no effect on serum or liver lipids.

Our data confirm previous studies in the rat reported by Kay and Truswell (8) and Randhotra (9), as well as studies in the hamster (10). Failure of bran to reduce blood lipid levels has also been reported in man in a number of studies (11–14).

The negative results of these studies utilizing bran do not necessarily imply that other components of dietary fiber lack a lowering effect on serum lipids. The subject continues to stimulate considerable interest and variable results have been reported with different types of vegetable fiber depending on the amount used, physicochemical properties, and the biological implication on bile salts and intestinal flora.

Summary. The effect of bran on diet-induced hyperlipidemia was examined in a long-term 16-week study in the rat. Animals were divided into four groups: group I (control diet with cornstarch); group II (cornstarch with 10% wheat bran); group III (atherogenic diet with sucrose); and group IV (atherogenic diet with sucrose and 10% wheat bran). The addition of bran did not affect serum and liver lipids (cholesterol and triglycerides) in the control or the experimental groups on atherogenic diet.

This work was supported by the Kaw Valley Heart Association Grant 9243, Kansas City, Kansas, the Kansas University Endowment Association Grant 2550, and General Research Support Grant 1522-14

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Received September 7, 1976. P.S.E.B.M. 1977, Vol. 154.

F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 553-557 (1977)

ɔcts of Interruption of the Ventral Noradrenergic Pathway on the Proestrous Discharge of Prolactin in the Rat (39717)

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ıction. Considerable evidence has to indicate the participation of ca-ıinergic pathways in the control of ·opin and prolactin release (1). Do-:learly functions as an inhibitor of release, either by causing the dis-f a peptidic prolactin-inhibiting fac-ı which then inhibits prolactin or by ʒ prolactin by a direct action on the er its release into hypophyseal por-ls. The evidence for a role for nor-ine in the control of prolactin has gmentary. Restoration of norepi- synthesis in animals in which cate-ıe synthesis had been inhibited by l paratyrosine resulted in a rise in ·rolactin, which suggested that nor-ine might play a stimulatory role to prolactin release (2). In later ex-:s, intraventricular injection of nor-ine prevented a decline in prolactin :curred in saline-injected controls, ɔuld also be consistent with a norep-ɛ-induced stimulation of prolactin 3). The cell bodies of most noradre-:urons which terminate in the hypo-region are located in the brain stem ısequently, in order to determine ɔrtance of noradrenergic pathways ntrol of gonadotropin and prolactin we have begun experiments in ese pathways are interrupted by in-ɔf 6-hydroxydopamine. This agent ly destroys catecholaminergic neu-

dy the effect of interruption of the noradrenergic pathway on the ɔf prolactin which occurs on proes-ydroxydopamine was injected into the ventral noradrenergic tract on the morning of proestrus and its effect on the proestrous discharge of prolactin was studied.

Material and methods. Adult virgin female rats of the Sprague-Dawley strain (Simonsen Laboratories, Gilroy, Calif.) were housed in group cages under conditions of controlled temperature (24°) and lighting (lights on 5:00 AM–7:00 PM). Only rats which had shown two or more consecutive regular 4- or 5-day cycles were used for experiments. The animals were given free access to water and Purina laboratory chow. On the morning of proestrus, the animals were placed in a stereotaxic instrument and 6-hydroxydopamine (2 μl containing 20 μg dissolved in 0.9% NaCl, solution acidified to pH 4.5 with ascorbic acid) was microinjected bilaterally into the ventral noradrenergic tract at the level of the interpeduncular nucleus and medial lemniscus (4). One-milliliter blood samples were withdrawn just prior to operation, at 5:00 PM, and on the following morning. The animals were lightly anesthetized with ether prior to bleeding.

At sacrifice, on the following morning, the hypothalamic region was rapidly frozen in liquid propane and freeze-dried for a period of 6 days. Afterwards, the freeze-dried hypothalami were treated with paraformaldehyde (70% relative humidity) at 80° during a period of 1 hr according to the Falck-Hillarp procedure (6, 7). All pieces were embedded in paraffin *in vacuo* and the blocks were sectioned in slices 10 μm thick. The fluorescence microscopy was performed with a Zeiss microscope equipped with a dark field condenser and a HBO-200 high pressure mercury lamp. The brain stems were fixed in 10% neutral formalin, cut in serial sections at 10 μm, and stained with hematoxylin-eosin to determine the localization of lesions. On the basis of the decline in hypothalamic catecholaminergic

ctoral Fellow of the Medical Research Canada.

ork was supported by grants from the NIH) and HD 05151) and the Ford Foundation.

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ISSN 0037-9727

fluorescence and the location of the lesions, the animals were grouped into those with complete lesions of the noradrenergic tract and those with incomplete lesions.

Results. In control animals there was an elevation of prolactin above the morning values on the afternoon of proestrus in every case, and the mean increase was highly significant (Table I). Lesions which nearly completely or completely interrupted the ventral noradrenergic tract on the basis of a study of their effect on the hypothalamic catecholamine fluorescence and/or th calization of their lesions in the brain blocked the afternoon elevation of prol in every case. Localization of the effe lesions in the region of the interpedun nucleus is illustrated (Fig. 1). In fac those animals with effective lesions by criteria the prolactin titers declined i afternoon of proestrus in every animal the decline was highly significant.

In another group, the lesions were e misplaced, or asymmetrical and were as

TABLE I. THE EFFECT OF LESIONS IN THE VENTRAL NORADRENERGIC TRACT ON THE PROESTROUS DISCHARGE OF PROLACTIN.

	Plasma prolactin (ng/ml)				
	AM	PM	Difference	Fluorescence	Lesions
Controls (N = 13)[a]	62 ± 7.6[b]	139 ± 19	79 ± 21*		
Effective lesions (N = 13)					
B-12	89	15	−74	MD[c]	+[d]
8A	37	4	−33	MD	+
19A	133	55	−78	0[e]	+
23A	80	13	−67	0	n.a.[f]
101A	50	8	−42	n.a.	+
119A	43	7	−36	0	n.a.
126A	52	11	−41	0	+
102A	28	6	−22	n.a.	+
106A	37	5	−32	MD	+
109A	58	3	−55	0	+
123A	21	2	−19	MD	n.a.
125A	29	10	−19	n.a.	+
127A	51	18	−33	0	+
	54 ± 8.5	12 ± 3.8	−42 ± 5.6**		
Incomplete lesions (N = 9)					
B1	40	45	5	n.a.	Too far lateral
B4	25	77	52	−[g]	Too far lateral dorsal
B11	59	90	31	−	Asymmetric dorsal
B8	75	81	6	n.a.	Asymmetric dorsal
134A	79	160	81	−	n.a.
150A	59	55	−4	−	n.a.
172A	24	103	79	−	Asymmetric; too lateral
141A	48	40	−8	−	n.a.
154A	30	58	28	Almost normal	Asymmetric
	49 ± 6.9	79 ± 12	30 ± 11***		

[a] N, number of rats.
[b] Mean ± SE.
[c] MD, marked decrease in hypothalamic catecholaminergic fluorescence.
[d] +, properly placed 6-OHDA lesions.
[e] 0, absent hypothalamic catecholaminergic fluorescence.
[f] n.a., not available for study.
[g] −, slight or asymmetric decrease in hypothalamic catecholaminergic fluorescence.
* $p < 0.01$.

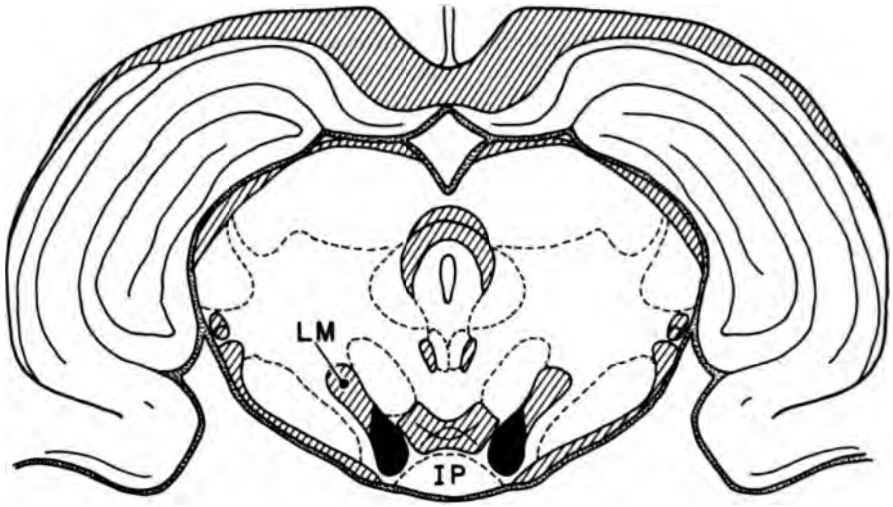

FIG. 1. Location of areas of destruction common to effective lesions in the ventral noradrenergic tract which are seen to lie within the medial lemniscus (LM) just dorsal and lateral to the interpenduncular nucleus (IP).

TABLE II. THE EFFECT OF LESIONS IN THE VENTRAL NORADRENERGIC TRACT ON THE PROESTROUS DISCHARGE OF LH.

	Plasma LH (ng/ml)		
	AM	PM	Difference
Controls (N = 13)[a]	3.6 ± 0.9[b]	43.1 ± 9.3	41.8 ± 10.0*
Effective lesions (N = 13)	2.6 ± 0.5	4.7 ± 1.4	2.1 ± 1.3
Incomplete lesions (N = 9)	5.5 ± 1.0	4.4 ± 1.3	1.2 ± 1.3

[a] N = number of rats.
[b] Mean ± SE.
* $p < 0.01$.

ated with little or no decrease in the catecholamine fluorescence. In these cases, the situation was intermediate between that in the control animals and that in those with effective lesions. The afternoon prolactin values were increased above those in the morning in all but two of the nine animals, but the increase was small except in four cases. The mean increase was significant statistically ($p < 0.05$).

There was an increase in plasma LH in all of the control animals on the afternoon of proestrus in comparison with the values during the morning (Table II). This increase was blocked by the effective lesions but was also blocked by the ineffective lesions, which did not produce a complete block of the rise in prolactin.

Discussion. The results of the present study indicate that effective lesions of the ventral noradrenergic tract which would diminish the release of norepinephrine from the axon terminals within the hypothalamus prevent the rise in prolactin which normally occurs on the afternoon of proestrus in the rat. These lesions appear to have a specific action since lesions which were misplaced or were asymmetrical were associated with some increase in the levels of this hormone. Similarly, these lesions blocked the increase in LH which occurs on the afternoon of proestrus; however, in this instance, the poorly placed lesions were also effective, which raises the question as to whether or not the inhibition was a specific one. Subsequent experiments, in which the effect of these lesions on the progesterone-induced LH release in ovariectomized, estrogen-primed animals was studied, suggest that this is a specific effect since, in that series of experiments, injections of the ascorbic acid diluent into the ventral noradrenergic tract

did not block the rise in LH (8).

A hypothesis which can explain the ability of these lesions to block the proestrous discharge of gonadotropins and prolactin is that on the afternoon of proestrus there is an increased firing rate of neurons of the ventral noradrenergic tract which project to the preoptic anterior hypothalamic region. These neurons synapse there with LH-releasing hormone (LHRH) neurons in the case of LH and prolactin-releasing factor (PRF) neurons in the case of prolactin to stimulate a discharge of these neurohormones, which then provokes the rise in LH and prolactin, respectively. Indeed, prolactin-releasing activity has been extracted from the suprachiasmatic region (9), a region which is rich in noradrenergic terminals, presumably derived largely from the ventral noradrenergic tract (4, 10, 11). Similarly, in the case of LHRH, noradrenergic terminals presumably end in juxtaposition to LHRH neurons in the preoptic anterior hypothalamic area. Inhibitors of catecholamine synthesis have been shown to block the rise in plasma LH following electrochemical stimulation of the preoptic anterior hypothalamic region (12), and this blockade is reversed by administration of drugs which restored norepinephrine but not dopamine synthesis.

In the case of prolactin, which is under dual hypothalamic control by both PRF and PIF, it is also possible to explain the results on the basis of noradrenergic inhibition of PIF release. Further work will be necessary to distinguish between these two possibilities.

It must be recognized that these are acute lesions and that the 6-hydroxydopamine injections destroyed other neurons in addition to the fibers of the noradrenergic tract (13). Consequently, we cannot be certain at the present time that the results are due to the deficiency in norepinephrine which was produced by the lesions. The results are presumably not related to interruption of dopaminergic axons since dopaminergic terminals within the hypothalamus arise from the intrahypothalamic tuberoinfundibular dopaminergic tract (4, 10) which would not be affected by these brain stem lesions. Additional studies with lesions in other loci along the ventral noradrenergic tract a chronic lesions to eliminate the stress effects of acute lesions are ne

An alternate hypothesis to exp results would be that tonic dischar ventral noradrenergic pathway is to maintain the central excitatory the PRF neurons and that the fa prolactin release following destru the tract is simply related to the central excitatory state. Again, fu perimentation will be necessary t mine whether an actual increase in transmission in the ventral norad tract occurs on the afternoon of pro whether tonic activity in this tract i is required for the release of prola LH to occur on proestrus.

Completeness of lesions was ju their location in a region known to the noradrenergic tract at the leve interpeduncular nucleus (4). In a the decline in cathecholaminergic cence was used as an index of comp of lesions. It is realized that this is n titative and some reduction in the cence could be missed; however, w fluorescence is dramatically reduce sent, we believe this is certainly an i interruption of the tract. Furtherm subsequent experiments, we have measured a reduction in norepinep the hypothalamus following simila droxydopamine-induced lesions (Ma and McCann, unpublished).

Summary. 6-Hydroxydopamine croinjected bilaterally into the vent adrenergic tract at the level of the duncular nucleus. Effective injectic the tract as indicated by the lesions and by a decline in the catecholami resence in the anterior hypothalam associated with a complete blockad rise in prolactin which occurs on th noon of proestrus and, in fact, a de prolactin ensued. Lesions which wer metrical or missed the ventral noradr tract had only a slight effect in inhibi release of prolactin on the aftern proestrus. All lesions were capable o ing the release of LH on the afterr proestrus. It is postulated that increa pulse traffic along the ventral noradr

act induces the proestrous discharge of olactin and LH or that, alternatively, nic activity in this tract is required for this lease to occur.

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Received July 9, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 558-561 (1977)

Effects of Intravenous Infusion of TRH on Plasma TSH and Prolactin Concen in Rats (39718)

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Recent studies employing iv injection(s) of LH-releasing hormone (LHRH) have suggested that both endogenous (1) and exogenous (2) LHRH can prime the adenohypophysis to release an increased quantity of LH in response to additional stimulation with LHRH. In additional studies (3, 4) which support this view, two stages of pituitary LH and FSH release were clearly revealed by continuous constant-rate iv infusion of LHRH. Plasma LH and FSH concentrations rose a relatively small amount for approximately 40 min during LHRH infusion. This was followed by "augmented response" in which a rapid and marked increase in plasma LH and FSH concentrations occurred. The lag period of approximately 40 min could not be advanced by increasing the LHRH infusion rate, and the response did not require intact ovaries during infusion. These studies (3, 4) also revealed that a pituitary "refractory" mechanism exists, at least in the case of LH release. After a period of continued stimulation, the pituitary releases only minimal amounts of LH even though it contains substantial stores of LH and continues to be stimulated by LHRH.

It is of importance to determine whether release of other pituitary hormones can be governed by a "priming" and/or "refractory" mechanism. Synthetic TSH-releasing hormone (TRH) is readily available, and administration of TRH has been reported to increase plasma prolactin as well as TSH in female rats (5, 6). The results in male rats are conflicting (6-8). The present investigation was undertaken to determine whether a "priming" and/or "refractory" mechanism exists for pituitary TSH and prolactin release via the TRH stimulation mechanism.

Materials and methods. Sprague-Dawley (Simonsen) rats were housed in a room with the lights on from 0500-1900 hr. The stages of the estrous cycle were n by daily vaginal lavage and o which exhibited two or more conse or 5-day estrous cycles were use day cyclic rats (220-262 g) were proestrus or during the first day trus. Five-day cyclic rats were o mized and used 4 to 6 weeks later (g). Sixteen male rats (210-232 g) v used.

In all rats, two cannulas were into the right atrium of the heart 0530 and 0700 hr, as described p (3). Saline or TRH (Beckman, lot B saline was infused through one from 1100-1600 hr and blood (0.35 withdrawn through the other can mediately before and at various ti ing infusion by the techniques c previously (3).

Plasma TSH and prolactin c tions were measured by radioim say, with use of the kits and ins provided by NIAMDD. Values pressed as nanograms per mill plasma in terms of the NIAMD dards, which have biological p equivalent to 0.22 USP of (bovine) mg and 11 IU/mg for TSH and p respectively. Student's t test (samples) was used to analyze the d value < 0.05 was considered to b ceptable level of significance.

Although TRH was diluted in s line immediately prior to all infusi possibility existed that the TRH n compose significantly over the infu riod. The following bioassay sugg no significant decomposition too Additional rats on the first day of were fitted with a single indwellin cannula and were injected with 10 ng of TRH with a freshly made TF tion or a TRH solution which was to stand at room temperature for

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injection. The rises in plasma TSH
ntration after injection of the two
preparations at either dose level
not significantly different from each
and were similar to the values plot-
Fig. 4 of a previous paper (5). Four
/ere used for experimentation in each
four groups.

ults. Effects of TRH on plasma
In rats on the first day of diestrus,
on of 1 μg of TRH/hr caused a
:d elevation ($P < 0.001$) in plasma
concentration within 15 min (Fig. 1,
Mean plasma TSH concentration re-
:d high and showed no significant
e over the next 105 min. Plasma
declined during the next 3 hr of infu-
out was still significantly elevated (P
)01) at the end of the 5-hr infusion
l. Infusion of TRH at the 100 ng/hr
lso produced a significant ($P < 0.01$)
ise in plasma TSH concentration.
ver, plasma TSH in the 100 ng/hr
remained at elevated levels around
teau for the entire 5-hr infusion pe-
Plasma TSH concentration remained
constant and did not change signifi-
for the 5-hr period in saline-infused
nd in rats infused with 1 or 10 ng of
hr. The TSH values in the groups of
iven saline or 1 ng of TRH/hr were
ined to facilitate plotting of the data.
similar patterns in plasma TSH con-
ition were seen in rats infused with
or the different dosages of TRH in
rous rats (top of Fig. 2) and in four
ctomized and four male rats (not il-
ted).

:cts of TRH on plasma prolactin.
a prolactin concentration showed
fluctuations during infusion of sa-
r 1, 10, or 100 ng of TRH/hr in rats on
rst day of diestrus but no statistically
cant differences were measured dur-
e 5-hr period (Fig. 1, bottom). On the
hand, infusion of 1 μg of TRH/hr
l a small but statistically significant
se ($P < 0.05$) in plasma prolactin,
ily at 15 and 30 min after the start of
on. Similar patterns in plasma pro-
concentration were seen in proes-
rats during the first 2 hr of infusion
t that the brief elevation in plasma

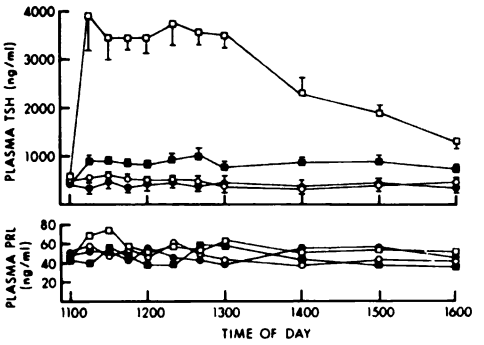

FIG. 1. Mean plasma TSH and prolactin concentrations in rats on the first day of diestrus. Rats were infused with saline or 1 ng of TRH/hr (shaded circles), 10 ng of TRH/hr (unshaded circles), 100 ng of TRH/hr (shaded squares), or 1 μg of TRH/hr (unshaded squares) from 1100–1600 hr. Plasma TSH and prolactin were measured on the same samples. Each group consisted of four rats. Standard errors are not plotted on mean TSH values at 1100 hr and on all mean PRL values to make display of the data more clear.

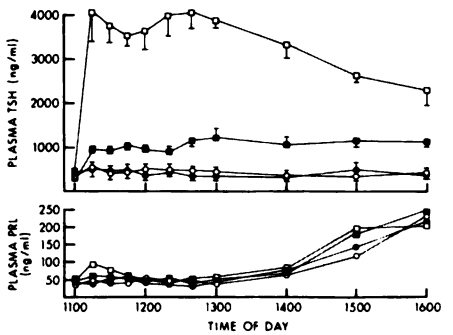

FIG. 2. Mean plasma TSH and prolactin concentrations in proestrous rats. See legend to Fig. 1 for details.

prolactin in the 1 μg/hr group was more pronounced ($P < 0.05$) at 15 min after the start of infusion (Fig. 2, bottom versus Fig. 1, bottom). Within 3 hr of infusion plasma prolactin concentration had started to rise and it was markedly elevated ($P < 0.001$) at 1500 and 1600 hr in all groups of proestrous rats (Fig. 2, bottom). Plasma prolactin was not elevated after the start of infusion of TRH at 1 μg/hr in both ovariectomized and male rats (not illustrated).

Discussion. The results of this study strongly indicate that TRH does not prime the adenohypophysis to release increased amounts of TSH or prolactin in response to

additional stimulation by TRH. Maximal or near-maximal levels of plasma TSH and prolactin were reached within 15 or 30 min after the start of infusion of TRH at 1 μg/hr and, in the case of TSH, at the 100 ng/hr infusion rate as well. Infusion of TRH at lower rates did not elevate plasma TSH or prolactin initially, and these hormones did not rise in plasma after continued infusion. Since TRH failed to "prime" the pituitary in females with widely different steroidal background milieu and in males, it is unlikely that TRH can prime the pituitary to itself under other *in vivo* conditions.

The rise in plasma prolactin concentration during TRH infusion at 1 μg/hr was similar to the rise seen after a rapid injection of TRH. The rise in the cyclic rats was small and brief. The fact that plasma prolactin did not remain elevated very long, even though the infusion of TRH was continued, indicates that either only a small amount of the total radioimmunoassayable prolactin in the pituitary can be released by TRH and that this pool is not replenished rapidly, or that some factor released during TRH infusion is blocking TRH-induced prolactin release. Were it not for the failure of saline and low dosages of TRH to elevate plasma prolactin during infusion, one might consider the TRH-induced prolactin release to be only a "stress" response. Nevertheless, it is possible that some (9, 10) but not all (5) types of stress- or drug-induced prolactin release may be mediated by TRH release. In any event, prolonged exposure of the pituitary to TRH did not interfere with the onset or magnitude of the spontaneous prolactin surge on proestrus.

Since plasma prolactin did not rise in ovariectomized and male rats infused at the 1 μg/hr rate, it is suggested that estrogen may facilitate TRH-induced prolactin release. The fact that the increase in plasma prolactin was greater in proestrous than in diestrous rats supports this view since circulating estrogens are highest during proestrus in this strain of rat (11). It is possible that had TRH been infused at considerably higher rates, an increase in plasma prolactin might be measured in ovariectomized and male rats. Th for a high dosage of TRH to elevate prolactin in males has been pointed Mueller *et al.* (6).

Although all releasing hormones prime the pituitary to themselv shown in the present study, the should not be interpreted to mea other prolactin-releasing hormon other releasing hormones in addi LHRH may not produce priming eff pituitary cells. On the other hand, very well be that the LHRH mechanism is unique. Althoug LHRH priming response was induc perimentally, it now appears to be o iological significance. The evide strong that the preovulatory sur plasma LH and FSH which start the afternoon of proestrus involve of the pituitary to additional LH LHRH itself (3, 4).

Plasma TSH concentration rose to high levels and was maintaine plateau until at least 2 hr after the infusion of TRH at 1 μg/hr. The there was a diminution of TSH rel response to presumably constant blo els of TRH. This decrease was sim the decline in plasma LH after a mately 3.5 hr of continuous LHR sion at a rate which simulated the trous LH surge (3). In the case of was demonstrated that the pituita fractory mechanism did not appear the result of a depletion of total ra munoassayable pituitary LH stores gonadal feedback mechanism (3). Si tuitary TSH concentration was not sured and since TRH was not infu thyroidectomized rats, it is not poss ascertain whether the decline in TSH concentration is a reflection duced pituitary TSH stores or the re a negative feedback mechanism of t hormone(s) on the adenohypophysi ternatively, the decrease in plasma could be due to some other mechani in the case for LH release.

In contrast to pituitary refractorin LHRH-induced LH release which p role in ending the preovulatory su LH in plasma (3), the physiological

cance, if any, of pituitary refractoriness to TRH-induced TSH release remains to be determined. Studies utilizing ultrastructural immunocytochemical techniques coupled with measurements of pituitary TSH content in normal and thyroidectomized TRH-infused rats are underway to elucidate the mechanism responsible for decreased pituitary TSH output.

Summary. In rats, blood was withdrawn through one of two indwelling atrial cannulas while saline or TRH in saline was infused at a constant rate for 5 hr through the other. Plasma TSH and prolactin were measured by radioimmunoassay. Infusion of TRH at 100 ng or 1 μg/hr in ovariectomized and male rats as well as 4-day cyclic rats on proestrus and the first day of diestrus caused a rapid rise in plasma TSH which remained at a plateau for 5 and 2 hr, respectively. In the 1 μg/hr group, plasma TSH declined progressively during the last 3 hr of infusion. No increases in plasma TSH were measured during the 5 hr period after infusion of saline or TRH at 1 or 10 ng/hr. Plasma prolactin rose a small but significant amount only in cyclic rats infused at the 1 μg/hr rate but it decreased to basal values after 30 min of infusion even though infusion was continued. The results suggest that TRH does not prime the pituitary to release greater quantities of TSH or prolactin in response to additional stimulation by TRH. They also suggest that a pituitary refractory mechanism for TRH-induced TSH release exists, but it remains to be determined whether any physiological significance can be attributed to this refractory mechanism.

The author thanks the Rat Pituitary Hormone Program, NIAMDD, for the generous gifts of materials used in the radioimmunoassay procedures in this study, and Sue Zurek for the typing of the manuscript. This study was supported by a grant from the University of Nebraska Medical Center Foundation.

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Received November 15, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 562-567 (1977)

The Effect of Sodium Depletion on the Renal Response to Short-Duration NH_4C Loading (39719)

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Introduction. Current concepts of the renal control of external hydrogen ion balance emphasize the importance of the relationships between the delivery of sodium to the distal nephron, the avidity of the distal exchange sites for sodium, and the excretion of poorly permeant anions (1). Sodium depletion would be expected to have two independent and opposing influences on renal acidification. Although the distal delivery of sodium might decline, distal sodium for hydrogen exchange would presumably be facilitated (2). There have been very few systematic studies of the influence of sodium depletion on renal acidification. To our knowledge the only study in humans in which volunteers were acid loaded both prior to and after sodium depletion showed an impairment in ammonium excretion (without change in urine pH) in the latter case (3). On the other hand, Schwartz *et al.* (4) found that sodium-depleted acid-loaded subjects excreted ammonium at a rate greater than that reported by others for normal sodium-replete subjects. We present here our findings in five normal subjects who underwent short-duration NH_4Cl acid-loading tests both before and after the induction of mild sodium depletion. The results suggest that sodium depletion limits maximal distal hydrogen ion secretion in response to acid loading.

Materials and methods. Five healthy male volunteers aged 20–25 years, hospitalized at the Clinical Research Center, University of Miami School of Medicine were evaluated. None gave a history of renal or cardiovascular disease or was receiving any medications. Creatinine clearance and serum sodium, potassium, chloride, and bicarbonate concentrations were normal (Tal There was no glucosuria or proteinu there were no abnormalities in the sediment. Urine cultures showed no in all subjects.

After admission the subjects were diet containing 150 mEq of sodium mEq of potassium daily for 3 days. C 4 the subjects underwent a short-d NH_4Cl acid-loading test. Immediatel the acid-loading test the patients started on a 10-mEq sodium, 60-ml tassium diet until Day 8 when the test was repeated. A single dose of fu ide, 20 mg by mouth, was given the m following the first acid-loading tes subjects had access to unrestricted a of distilled water; daily activities we limited. Body weight, blood pressure and standing, and 24-hr urinary sodiu tassium, and creatinine excretion wer sured daily throughout the study.

Acid loading. The evening prior study, the subject ate his usual me then had no food until the protoc completed, but was allowed water i amounts. He remained recumbent a as possible after awakening on the the study. All urines were obtained b taneous voiding and collected under oil. At 7:00 and 8:00 AM (baseline men) the subject voided. At 8:00 A rial and venous blood samples were for baseline measurements. In obtain venous sample, blood for serum dete tions was drawn first into a 20-ml with the tourniquet being released i ately to avoid stasis. A 2-ml hepa syringe was then transferred to the for aspiration of the blood sample and PCo_2 measurements. The subje then given 1.9 mEq/kg body wei NH_4Cl prepared in gelatin capsules gested with a total of 200 ml of wat

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TABLE I. EFFECT OF SODIUM DEPLETION ON SERUM AND URINARY ELECTROLYTES.

Weight (kg)	$U_{Na}V$[a] (μEq/min)	U_KV[a] (μEq/min)	4-Day cumulative urinary balance Na (mEq)	4-Day cumulative urinary balance K (mEq)	Serum Na (mEq/liter)	Serum K (mEq/liter)	Serum HCO_3 (mEq/liter)	Serum Cl (mEq/liter)	Ccr[b] (ml/min)
78.3±6.3[c] (69-101)	161±5 (153-179)	63±6 (42-77)	–	–	140±1 (138-141)	4.4±0.2 (3.9-4.9)	27±2 (21-30)	104±1 (100-107)	138±9 (121-171)
<0.001	<0.001	NS[d]	–	–	NS	NS	NS	<0.05	<0.025
76.6±6.4	7±3	57±5	−213±34	+11±8	138±1	4.2±0.1	28±1	101±1	125±12
(67-99)	(1-16)	(43-70)	(−135--323)	(−5-+32)	(135-143)	(3.6-4.4)	(25-31)	(100-105)	(103-170)

iod preceding acid loading.
three to five periods during acid loading.
. Ranges are within parentheses.
ificant ($P > 0.05$).

itely a 2-hr period. Urines were ıourly for 6 hr after ingestion of ıad. Venous blood was drawn 2 hr 6 hr after the acid load. Fifty of water per hour were given by ɔ sodium-free candy provided as ıring the study.

consent was obtained after explathe details of the procedures and ial risks. The research was carried ling to the principles outlined in ration of Helsinki and was apy the Human Experimentation es of the University of Miami and Veterans Administration Hospiverse effects occurred.

ination of the pH and PCo_2 of urine was completed within 5 min on on a Radiometer Acid-Base 1. All urine and venous blood ere measured for concentrations , potassium, phosphorus, creatitotal solutes. Urinary titratable A) and ammonium (NH_4) content neasured. None of the urine specɔd contained glucose or abnormal f protein. The methods and calcuıployed are those previously dem this laboratory (5, 6). The data ıted as means ± SE and were statistically by means of Student's aired values (7).

Achievement of sodium depletion

As a result of the sodium-reɔt, the initial NH_4Cl acid loading (3), and furosemide administration, each of the five patients developed mild to moderate sodium depletion. This was manifested by an average weight loss of 1.7 ± 0.1 (SE) kg, cumulative urinary sodium loss of 213 ± 13 mEq up until the day prior to the second acid-loading test, the excretion of 7 ± 3 mEq of sodium during the 24-hr period prior to restudy, and a 9% decrease in endogenous creatinine clearance. Urinary potassium balance was unchanged. The serum concentrations of Na, K, and HCO_3 did not change significantly as a result of sodium depletion. The serum chloride concentration was slightly but significantly lower after sodium depletion.

Response to acid loading (Table II). There were no significant differences between the baseline arterial pH (before, 7.40 ± 0.01; after, 7.42 ± 0.02) or venous pH (before, 7.31 ± 0.02; after, 7.31 ± 0.01), or arterial HCO_3 (before, 25 ± 1; after, 28 ± 1 mEq/liter) or venous HCO_3 (before, 27 ± 2; after 28 ± 1 mEq/liter) values prior to and following sodium depletion. On both occasions acid loading resulted in significant and approximately equivalent decreases in venous pH (minimum before, 7.25 ± 0.01; after, 7.27 ± 0.01) and HCO_3 (minimum before 22 ± 1; after 22 ± 1 mEq/liter).

After sodium depletion minimal urine pH was higher (average increment 0.43 ± 0.07; range, 0.26 to 0.62; $P < 0.001$) in each subject, and maximal $U_{NH_4}V$ was lower ($P < 0.02$) following NH_4Cl acid loading (Table

TABLE II. URINARY CHANGES FOLLOWING ACID LOADING.

Patient		UpH[a]	$U_{NH_4}V$[a] (μEq/min)	$U_{NH_4}V$[b] (μEq/min)	$U_{TA}V$[a] (μEq/min)	$U_{Na}V$[c,d] (μEq/min)	U_KV[d] (μEq/min)	UpV[d] (μg/min)
1	B[e]	4.60	67	50	–	308	92	730
	A[f]	5.00	47	41	–	30	66	530
2	B	4.65	50	46	33	260	114	410
	A	5.21	39	28	–	12	48	270
3	B	4.61	47	36	33	253	109	720
	A	4.90	46	44	31	109	96	490
4	B	4.79	78	58	38	206	121	920
	A	5.05	67	46	43	79	167	730
5	B	4.59	67	46	35	306	114	860
	A	5.21	47	38	25	28	124	490
Mean ± SE	B	4.65 ± 0.04	62 ± 6	47 ± 3	35 ± 1	297 ± 19	110 ± 5	728 ± 88
P Value		<0.005	<0.02	NS[g]	NS	<0.005	NS	<0.01
	A	5.07 ± 0.06	49 ± 5	39 ± 3	33 ± 5	32 ± 12	100 ± 21	502 ± 73

[a] Maximal or minimal values.
[b] Six-hour average after NH_4Cl.
[c] The relatively elevated $U_{Na}V$ in some patients most likely reflects the natriuretic effect of NH_4Cl (3).
[d] At the time of minimal UpH.
[e] B, before.
[f] A, after.
[g] NS, not significant ($P > 0.05$).

II). Although the average $U_{NH_4}V$ for the 6 hr following acid loading was also lower, this difference did not achieve statistical significance. Because of a technical problem, titratable acid excretion rates were available in three subjects only and were not different before or after sodium depletion.

Figure 1 contrasts the increments in minimal urine pH associated with sodium depletion obtained in the present study (open circles) with the variable changes found in 21 repeated acid-loading studies performed in our laboratory in normal subjects and in patients with the incomplete syndrome of distal renal tubular acidosis (closed circles) restudied without changing the dietary sodium intake. In contrast with the mean increment in urine pH of 0.43 induced by sodium depletion, the mean change in minimal urine pH in the other group was −0.003 (range, +0.33 to −0.30).

At the time of minimal pH, $U_{Na}V$, U_pV, and V were significantly lower after sodium deprivation, while U_KV was similar (Table II). Likewise, there were no differences in urine osmolality (before, 677 ± 68 mOsm/kg of H_2O; after, 625 ± 91 mOsm/kg of H_2O). The average $U_{Na}V$ for the 6 hr of the acid-loading study was lower ($P < 0.01$) and the percentage of tubular reabsorption of phosphorus at the time of minimal urine pH was higher ($P < 0.05$) after sodium depletion (before sodium depletion, 195 ± 38

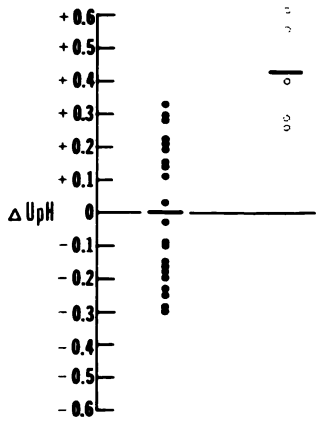

FIG. 1. Changes in minimal urine pH in r
acid-loading studies. Minimal urine pH was hig
erage increment, 0.43 ± 0.07) in each of
subjects reevaluated during sodium depletio
circles). Of 21 repeated short-duration acid
tests obtained in normal volunteers and in patie
the incomplete syndrome of distal renal tub
dosis (closed circles), 10 demonstrated incre
11 demonstrated decreases in minimal urine p
age change, −0.003 ± 0.05; $P < 0.01$ in comp
sodium depletion data).

μEq/min and 85.6 ± 0.7%, respec
after sodium depletion, 18 ± 8 μE
and 88.4 ± 0.8%, respectively). Th
age 6-hr phosphorus and potassium
tion rates did not differ at the time
two studies.

When $U_{NH_4}V$ was plotted against cc

itant urine pH before and after sodium depletion (Fig. 2), there was considerable overlap of the points. Calculation of the equations for regression lines revealed slopes which differed significantly from zero but not from each other, indicating that for a given change in urine pH the change in $U_{NH_4}V$ did not differ in the two studies. In addition, the lines did not differ from each other in elevation.

Discussion. In the present study the average minimal urine pH was higher and the maximal rate of ammonium excretion was lower after modest sodium depletion was induced in five normal volunteers. Although the changes noted were quantitatively small these observations support the concept that alterations in sodium metabolism may affect urinary acidification (1).

The higher minimal urine pH during sodium depletion could not be explained by changes in blood acid-base status. Urine flow rate and urinary phosphorus excretion were both lower following sodium depletion but these changes would not be expected to elevate urine pH (8, 9). In our laboratory, the minimal urine pH after short-duration acid loading has a good reproducibility and in general is likely to be either slightly greater or less than the initial value (average change in urine pH, -0.003 ± 0.05), in marked contrast to the present study in which all five subjects had increments in minimal pH of 0.26 or greater (Fig. 1).

In addition to an increase in minimal urine pH, sodium depletion was associated with a decrease in ammonium excretion. This change may have been due largely to changes in urine pH, since, when $U_{NH_4}V$ was plotted against urine pH, the equations for the regression lines were not different either in slope or elevation (Fig. 2).

Potassium loading is known to decrease urinary ammonium excretion (10). Our subjects manifested comparable serum potassium concentrations and urinary potassium excretion rates during the two acid-loading studies, and cumulative urinary potassium balance in between the studies was close to zero, suggesting that important changes in potassium status did not occur during the sodium-depleted state.

Aldosterone secretion is enhanced during sodium depletion and this might be expected to facilitate renal acid excretion (11, 12). It is known, however, that the increased acid excretion often associated with states of mineralocorticoid excess can be prevented by dietary sodium restriction (13). Moreover, limitation of sodium delivery to the distal tubule might be expected to result in a decreased rate of sodium reabsorption in the distal nephron, thus decreasing the negative potential necessary to promote hydrogen ion secretion (14).

Although renal tubular hydrogen ion secretion may be linked somehow to sodium reabsorption, the relationship is not believed to be the result of direct coupling (15). When the avidity for distal tubular sodium-cation exchange is increased at a time when the distal delivery of sodium is adequate, then hydrogen ion secretion is greatly facilitated (2). This formulation appears to hold for explaining the renal mechanisms acting to increase acid excretion both

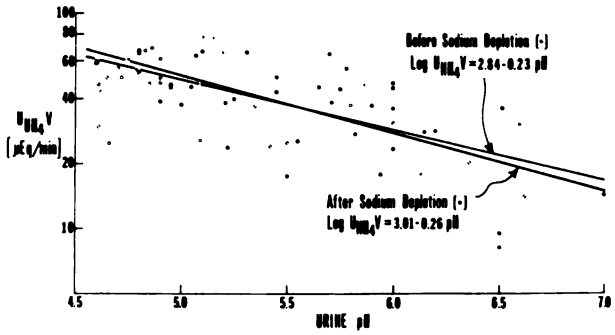

FIG. 2. Plot of $U_{NH_4}V$ versus urine pH before and after sodium depletion. The open circles denote values prior to and the closed circles denote those after sodium depletion. The regression lines and their equations are shown for each group. (See text for explanation.)

during mineral acid loading and during the development of metabolic alkalosis (1).

The effect of sodium restriction on sodium reabsorption in various nephronal sites is not well defined. We have no direct data regarding distal sodium delivery in our patients. There is experimental micropuncture evidence (16) that distal sodium delivery is reduced during mild sodium depletion. In addition, the data of Stein *et al.* (17) demonstrating that salt restriction limits Tc H_2O formation in normal subjects are consistent with decreased distal delivery of sodium. On the other hand, recent studies (18) have demonstrated that the collecting duct is primarily responsible for the increased sodium reabsorption during moderate extracellular fluid volume depletion.

One might expect that urine pH might be higher and acid excretion lower if the distal delivery of sodium is limited. This might be the case, at least in association with some types of disordered renal acidification. For example, Buckalew *et al.* (19) reported that the urine pH-lowering capacity of a patient with the incomplete syndrome of distal renal tubular acidosis was limited further by dietary sodium restriction and Better *et al.* (20) as well as ourselves (21) have suggested that the abnormal renal acidification associated with alcoholic liver disease is related at least in part to the concomitant sodium-retaining state. Furthermore, enhancement of distal sodium delivery by diuretic administration has ameliorated the acidification impairment in some patients with distal RTA (22).

There has been very little systematic evaluation of the influence of sodium depletion on the renal response to acid loading. Our findings are similar to those of Clarke *et al.* (3) in that these authors noted a lower ammonium excretion (without a change in urine pH) when NH_4Cl acid loading followed sodium depletion. Both of the patients studied by Ryberg during a 10-day acid-loading test excreted urine containing less acid and less ammonium during periods of low sodium excretion (23). The ingestion of 4% NaCl was promptly followed by enhancement of ammonium excretion and a lowering of urine pH. Sodium balance data was not provided but one might suppose that the sustained NH_4Cl loading had resulted in a *substantial negative sodium* balance (23). After acid loading sodium-depleted subjects for 2–3 days, Schwartz *et al.* (4) demonstrated a relative enhancement of ammonium excretion in comparison to that reported by other investigators in sodium-replete volunteers. Their subjects, however, were not studied prior to sodium depletion and it is conceivable that their ammonium excretion was increased by some other variable or that distal sodium delivery was facilitated at the time of acid loading.

The findings in the present study are consistent with reports stressing the importance of distal tubular sodium reabsorption in renal acid handling. Although we have no direct data in our patients relevant to the distal delivery of sodium, it is tempting to speculate that in our sodium-depleted subjects maximal distal hydrogen ion secretion was limited by decreased distal delivery of sodium despite presumed increase in sodium avidity at the same nephronal site.

Summary. The effect of sodium depletion on urinary acidification was investigated in five healthy male volunteers. The response to short-duration NH_4Cl loading was assessed both before and during sodium depletion induced by low salt diet plus a single dose of furosemide. The minimal urine pH was higher ($P < 0.005$) and the maximal rate of ammonium excretion was lower ($P < 0.02$) following sodium depletion. These changes could not be explained by changes in acid-base status, serum potassium concentration, urine flow rate, or urinary phosphorus excretion. The findings support the concept that sodium delivery to distal exchange sites in concert with sodium reabsorptive avidity are major regulators of the renal response to acid loading. It is suggested that following sodium depletion a decreased distal delivery of sodium limited maximal hydrogen ion secretion despite the probable enhancement of the avidity for H^+-Na^+ exchange.

The authors wish to express their appreciation to Kenneth Bailey, Raul Rodriguez, Robert Rubin, Lottie Scott, Joan Spiegel, Charlene Morgan, and Bonnie Agnell.

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Received November 1, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 568-572 (1977)

Chondrogenesis in Tissue Cultures of Muscle Under the Influence of a Diff Component of Bone Matrix (39720)[1]

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When neonatal muscle is cultured on a nonbiologic substratum such as glass, plastic, or cellulose acetate, the fiber bundles disintegrate while the connective tissue disaggregates and becomes mesenchymal cells, which migrate and proliferate. On a biologic substratum such as bone matrix (1) or bone matrix gelatin (2), or on the residue of collagenase-digested bone matrix gelatin (3), the mesenchymal cells reaggregate and differentiate not into fibrous tissue but into cartilage (4–6). On a control biologic substratum of bone matrix either partially digested with trypsin or on gelatin prepared from bone autodigested by endogenous enzymes in a buffer solution (7), the cells differentiate into fibrous tissue only (3–4). Through contact of mesenchymal cells with a hypothetical bone morphogenetic protein (BMP) in bone matrix, the pathway of development is switched from fibrous tissue to cartilage (2). The following experiments demonstrate transfer of BMP to mesenchymal cells through the nutrient medium without direct contact with bone matrix.

Methods. Samples of muscle were excised from the middle of the belly of the triceps humeri of 3-week-old Lewis strain rat fetuses. The muscle tissue was minced in a drop of culture medium CMRL 1066 containing 15% heat-inactivated newborn calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co.). The initial pH of the medium was 7.3. The minced muscle tissue wa on a chicken plasma clot and cellul tate membrane (Millipore Corp.), p 0.45 μm. The plasma-coated me was placed on a wire-grid platform i con organ culture dish (Falcon Plas vision of BD Laboratory, Los A Calif.). The culture was maintained an incubator, 5% CO_2 in air, Mod (National Appliance Co., Portland

Graduated quantities of the res bone matrix gelatin (BMG) (2) with purified collagenase (8) for o were placed in the space beneath t well *isolated from any possible cont* either the cellulose acetate membran cultured tissue. The assembly of the is illustrated in Fig. 1; the dosages c and the experimental results are rized in Table I.

The cultures were conditioned w cessively increasing quantities of ranging from 0 to 50 mg (25 mg of B of culture media). For controls, mu sue cultures were assembled: (a) any BMG in the space beneath the g with BMG prepared from autoc bone (7); (c) with BMG in the syst only for limited periods of time, from 1 to 7 days; (d) with living c bone. Duplicate cultures were trans on the 7th day into a muscle pouch syngeneic recipients.

The explants and transplants were neutral formalin on the 21st day. Hi cal sections were prepared and stain hematoxylin and eosin, azure II, an blue.

Results. Mesenchymal-like fibro nective tissue cells grew out of the ex muscle tissue by the seventh day and out in a thin layer on the surface cellulose acetate membrane (Fig. muscle fibers atrophied, and m either formed multinuclear aggreg

[1] These investigations were supported by grants-in-aid from the USPHS, National Institute of Dental Research (DE-02103-01), and the Leo Hulseman Foundation. Dr. M. Nakagawa received a research fellowship from the Solo Cup Corporation. The authors are indebted to Mrs. Susan Kirkpatrick, Mrs. Merci Rivera, and Ed King for excellent technical assistance.

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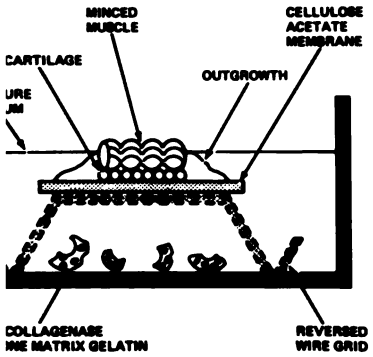
ISSN

ed in pyknotic fragments. By the in culture dishes containing 30 to BMG, cartilage cells differentiated e between the atrophied degener- le tissue and the surface of the acetate membrane. By the 21st ells were paired and enveloped in rtilage matrix. Azure II metachro- stained and alcian blue extracellu- ace extended into the pore open- distance of about 5 to 10 μm (Fig

ol culture dishes containing less ng of BMG, or BMG prepared ysed bone, or even living calvarial connective tissue outgrowths dif- l into fibrous tissue only without



Diagrammatic representation of tissue cul- y for outgrowth of mesenchymal cells of muscle onto a cellulose acetate membrane .45 μm).

any evidence of cartilage. When 50 mg of BMG were present for only limited periods of time from 1 to 6 days, cartilage developed on the 21st day (Table I). The yield of new cartilage at 21 days was somewhat less than when BMG was continuously present in the system. After 6 days in culture, the BMG disintegrated and could not be recovered. When the BMG was removed at earlier stages and transplanted into a muscle pouch in an adult rat, the matrix still had osteoinductive activity. The yield of new bone was much less than from unincubated BMG.

On the 7th day, with 50-mg doses of BMG beneath the grid, and spindle-shaped cells growing out on the membrane above the grid, the membrane and tissue culture attached to it were transplanted into a muscle pouch in an adult syngeneic recipient. Fourteen days later the membranes were covered with new cartilage and deposits of endochondral bone (Fig. 4).

Discussion. In the culture assembly described above, muscle connective tissue outgrowths differentiated into cartilage on a cellulose acetate membrane without any possible contact with BMG. The muscle explant was separated from BMG not only by a membrane but also by a wire grid and 2 ml of culture medium. Under these conditions, the medium would have had to transfer a soluble rapidly diffusible chondrogenetic molecule from the BMG to the explant. The

I. DEMONSTRATION OF CHONDROGENESIS AND OSTEOGENESIS EVOCATED IN EXPLANTS OF MUSCLE TISSUE BY DIFFUSIBLE COMPONENTS OF BONE MATRIX GELATIN (BMG).

Number of cultures with cartilage/total number of explants	Day BMG was withdrawn from culture	Product	Product of transplantation of 7-day-old cultures into isogeneic rats for 2 weeks
0/9	0	Fibrous tissue only	Fibrous tissue
0/6	0	Fibrous tissue only	Fibrous tissue
3/12	0	Cartilage	Bone
6/12	0	Cartilage	Bone
8/12	0	Cartilage	Bone
2/10	1	Cartilage	Bone
1/6	2	Cartilage	Bone
1/4	3	Cartilage	Bone
2/10	4	Cartilage	Bone
7/18	6	Cartilage	Bone
0/6	0	Fibrous tissue only	Fibrous tissue
0/6	0	Fibrous tissue only	Fibrous tissue
0/6	0	Fibrous tissue only	Fibrous tissue

epared from autodigested bone.

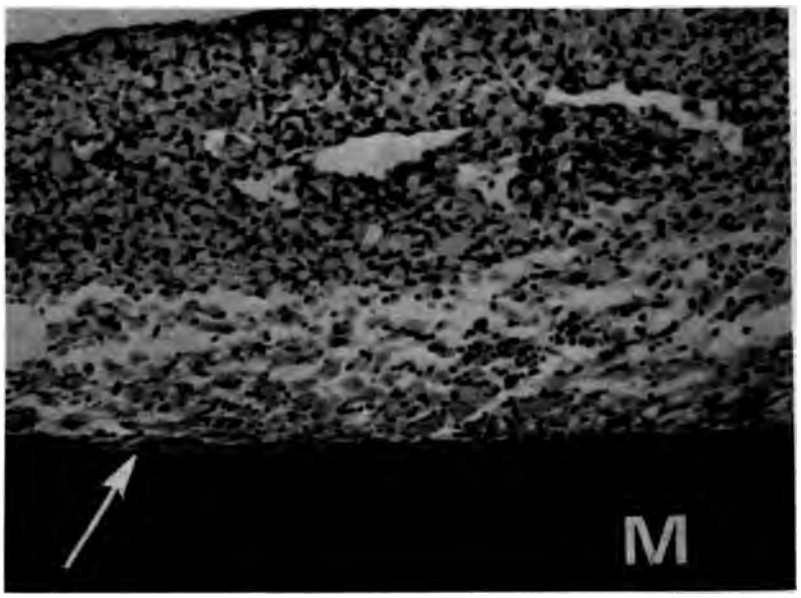


FIG. 2. Photomicrograph of a 7-day-old explant in the tissue culture assembly shown in Fig. 1. The indicates monolayer of mesenchymal cells derived from interfibrous connective tissue and spread out surface of the membrane (M).

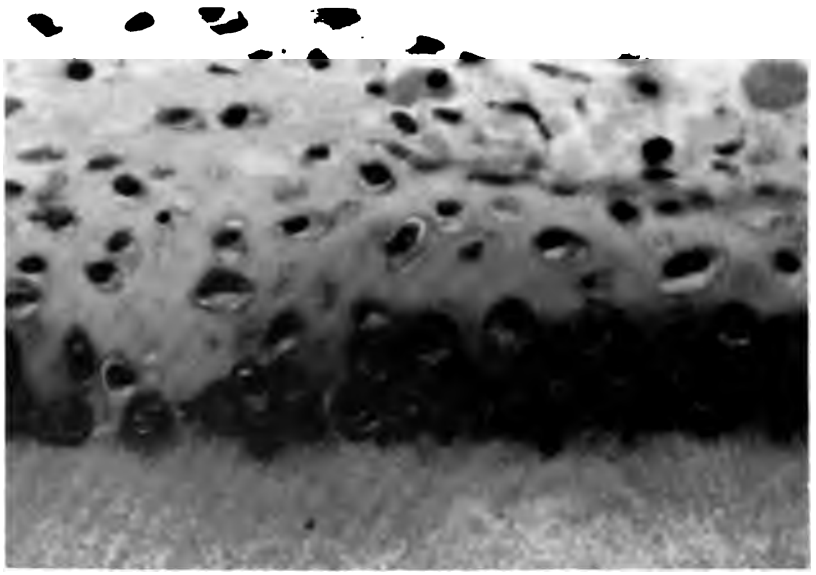


FIG. 3. Photomicrograph of a 21-day-old explant with hyaline cartilage from the outgrowth of interfibrillar connective tissue cells shown in Fig. 2.

transfer occurred even when the BMG was in the system only for the first 24 hr. The effect was bone morphogenetic as well as chondrogenic because, when the culture was transplanted into a syngeneic recipient, it differentiated into bone. Control cultures containing autolysed bone matrix or tured or living fully mineralized ca bone produced fibrous tissue only.

While close contact between cells a trix is characteristic of bone morphog systems under normal conditions (

FIG. 4. Photomicrograph of a transplant of 1 week-old-muscle tissue culture, along with its substratum of cellulose acetate, 2 weeks after transplantation into a muscle pouch in an adult syngeneic rat. Recipient muscle pouch (P); cartilage (C); new bone (B); cellulose acetate substratum (M).

under unusual conditions, i.e., in diffusion chamber (11) and in the tissue culture system described above, cartilage differentiation occurs without contact. Cartilage and bone morphogenesis occurs without cell contact with matrix in BMG-filled diffusion chambers having walls 300 μm in thickness and pore sizes of 0.025 to 0.45 μm (11). Even more striking, chondroosteogenesis occurs on the outside of the empty part of duplex chambers consisting of one chamber loaded with the residue of the collagenase digest of the bone matrix gelatin beside another filled with *interstitial fluid* percolating through from the surrounding recipient bed (12). The appearance of cartilage in avascular and bone in vascular environments in response to a rapidly diffusible BMG (2) suggests a complete change in phenotypic expression of the same genome (13).

The evidence that BMP is a protein is that it is trypsin labile at 15°, cathespin resistant, and hydrolyzed in 0.1 *N* NaOH at 2° within 12 hr without release of hydroxyproline (2). Moreover, BMP is collagenase resistant. The specific activity of BMP is high in BMG which is prepared by sequential chemical extraction of proteoglygcans, sialoproteins, and various other EDTA-soluble noncollagenous proteins (2). BMG is insoluble at 2°, but slowly soluble at 37° (2), and incorporates BMP in the so-called collagenase-released fraction of noncollagenous protein. Bone morphogenesis at the openings of infinitely tortuous pores of multiple-walled cellulose acetate membranes, which contain no EM-demonstrable fibrils or parts of fibrils (11), excludes the idea that BMP is derived from collagen. Whether BMP is a bone collagen extension peptide or a scission product of the bone procollagen molecule is presently under investigation.

Kosher *et al.* (14) observed the effects of proteoglycans and Solursh *et al.* (15) observed the effects of a culture medium conditioned with a trypsin-labile, heat-inactivated, mercaptoethanol-sensitive molecule, 30 to 150,000 daltons in weight. In both instances expression of cartilage differentiation was enhanced either in somite or limb-bud axial mesenchyme. BMP resembles this molecule as it is diffusible in a culture medium and operated by a positive feedback mechanism, but differs insofar as its target tissue is not embryonic organic anlagen but postfetal migratory mesenchymal cells.

When penetration of mammalian cells by diffusible molecules can be measured and their functions explored (16), knowledge of the composition and mode of action of the BMP is bound to emerge.

Summary. Under the influence of a diffusible molecule in bone matrix gelatin (BMG), connective tissue-cell outgrowths of neonatal rat muscle in tissue culture differentiate into cartilage. The muscle explant was suspended on a cellulose acetate membrane and separated from the BMG by a wire-grid platform and 2 ml of culture medium. Cartilage differentiated even when the BMG was removed from the system, within the first 24 hr of culture. Cultures transplanted on the seventh day into a muscle pouch in syngeneic recipients for an additional 14 days produced deposits of cartilage and woven bone.

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Received July 9, 1976. P.S.E.B.M. 1977, Vol

F THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 573-577 (1977)

# of Growth Hormone by TRH in Intact Rats or in Intact or Hypophysectomized Rats bearing a Heterotopic Pituitary (39721)

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uction. Thyrotropin-releasing hor- RH) is capable of releasing growth (GH) in several pathologic condi- man, e.g., acromegaly, uremia, lepression, and anorexia nervosa ıd in the laboratory animal espe- en the anterior pituitary (AP) lacks ıl neurohormonal influences (5-9). us to postulate that in the patho- ıditions of the human, the anoma- response to TRH also might be due ıistence of a functional disconnec- ween the central nervous system ıd the AP (8, 9). However, another y could not be excluded, i.e., that ıs in peripheral hormone titers due istence of the CNS-AP disconnec- ht induce a sensitization of the AP H-releasing effect of the tripeptide. ɔut this possibility in this study the f TRH to induce GH release was ł in either intact rats or in intact or sectomized rats bearing an AP graft e kidney capsule. In addition, elec- roscopy studies were performed on *u* or transplanted pituitary in basal ıs or following TRH stimulation.

als and methods. In all experiments and male Sprague-Dawley rats Erba S.p.A., Milano) were used. s of both sexes were hypophysecto- 0) and 10 days later they received e kidney capsule an entire AP ob- om rats of the same sex and weight Γ). Intact rats of both sexes also an entire AP under the kidney cap- ı. All experiments were performed ıfter transplantation in either HT or body weight, 120-140 g). As controls for both groups weight-matched intact female and male Sprague-Dawley rats were used.

Blood samples. Throughout all studies, blood samples (0.3 ml) were collected from the exposed jugular vein with rats under urethane anesthesia (1.5 g/kg body wt), with the exception of the first blood sample (0.4 ml) which was obtained in unanesthetized animals by puncture of the retro-orbital venous plexus (12). The second blood sample (0 min), was collected 60 min after urethane and immediately thereafter 0.2 ml of either normal saline solution or TRH (0.15, 0.6, and 1.2 μg) dissolved in saline was injected into the jugular vein. Five and ten minutes after injection blood samples were obtained from each rat and the blood removed was replaced with an equal volume of normal saline.

Hormone determinations. Blood samples were immediately centrifuged; the plasma was separated and kept frozen at −20°C until assayed. GH and PRL levels were measured by radioimmunoassay as previously described (13) employing materials generously supplied by the NIAMDD. All plasma samples for a given experiment were tested in a single assay to eliminate interassay variation in the interpretation of treatment effects.

Electron microscopy (EM) studies. EM studies were performed on 16 pituitaries from eight IT female rats (two from each dose group, randomly selected). Immediately after the last blood sample was taken, rats were killed and both the *in situ* and the ectopic pituitary were quickly removed and fixed in cold Karnowsky solution for about 4 hr and then washed in Sørensen buffer, 0.12 *M*, pH 7.4. From each pituitary a paramedial section of AP was obtained and divided in four portions, as described elsewhere (G.

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ISSN 0037-9727

L. Rossi *et al.*, in preparation). The portions of AP tissue were then embedded in Epon 312 as previously described (14) and blocks were properly positioned under a stereomicroscope. From one block randomly taken from each pituitary, ultrathin sections were cut, mounted on 300-mesh grids, and contrasted with uranyl acetate and lead citrate for EM. At × 14,000, three grid fields per block were examined and all figures of exocytosis found to occur in 10 somatotrophs randomly selected among those fullfilling the criteria elsewhere reported were counted.

Statistics. Absolute GH increases above pretreatment values observed after TRH injection were compared to those following saline administration. In all experiments the significance of differences was evaluated according to Duncan's multiple range test or analysis of variance (ANOVAR).

Results. Baseline GH values. No significant difference in plasma GH values was reported between intact and IT rats (49.9 ± 2.1 and 63.9 ± 13.3 ng/ml, respectively), while in accordance with previous results (8), plasma GH values were significantly lower in HT rats (9.0 ± 0.7 ng/ml). Urethane anesthesia significantly lowered plasma GH values in intact and IT (1 0.7 and 6.5 ± 1.1 ng/ml, respectively not in HT rats (8.7 ± 0.7 ng/ml).

GH response to TRH. Administrat TRH at doses of 0.15, 0.6, and 1 induced a significant rise in plasma G els in HT and IT female rats at both 10 min, except in IT rats with the two doses at 10 min. The GH rise after 1.2 TRH was more consistent in the IT t the HT rats ($F = 7.50$, $P < 0.05$) (I mid and right sections). In the intact f rats, of the three TRH doses used, or highest (1.2 μg) induced a significa crease in plasma GH at both 5 and 1(However, this dose was less effective t IT rats ($F = 8.82$, $P < 0.05$) (Fig. section).

A similar trend was present in mal in which the two doses of the trip used, 0.15 and 0.6 μg, increased p GH levels in HT and IT rats at both 10 (Fig. 2, mid and right sections). In rats TRH was effective as a GH-re only at the dose of 0.6 μg (Fig. 2 section).

Electron microscopy studies. EM ob tions showed that in IT rats the ectop tuitary had undergone the known ch

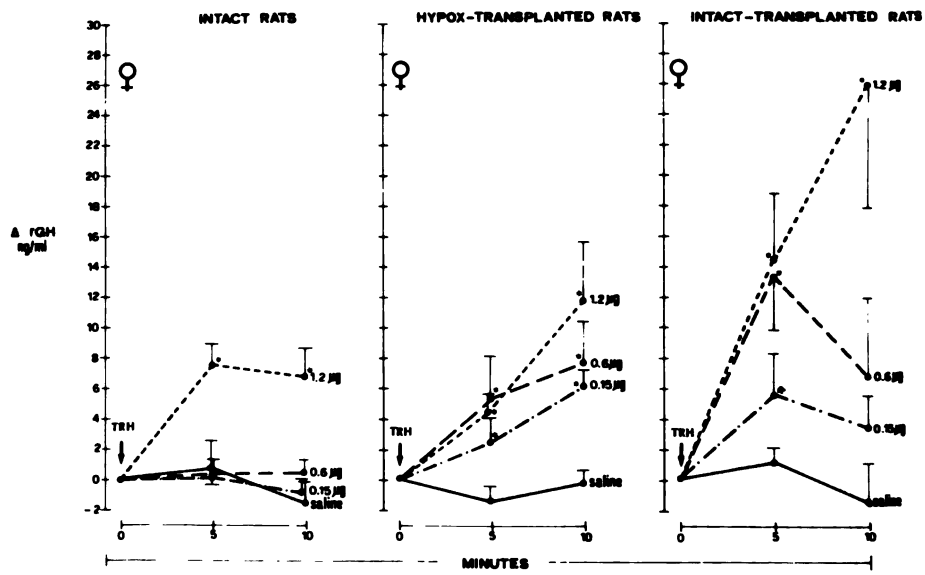

FIG. 1. GH-releasing effect of TRH in intact female rats, hypophysectomized rats with pituitary graf intact rats with pituitary grafts. Six to seven animals per group were used. Data are expressed as Δ values (n SEM) from baseline; asterisks denote a difference statistically significant from corresponding Δ values of treated rats (Duncan's test). The same description applies to Fig. 2.

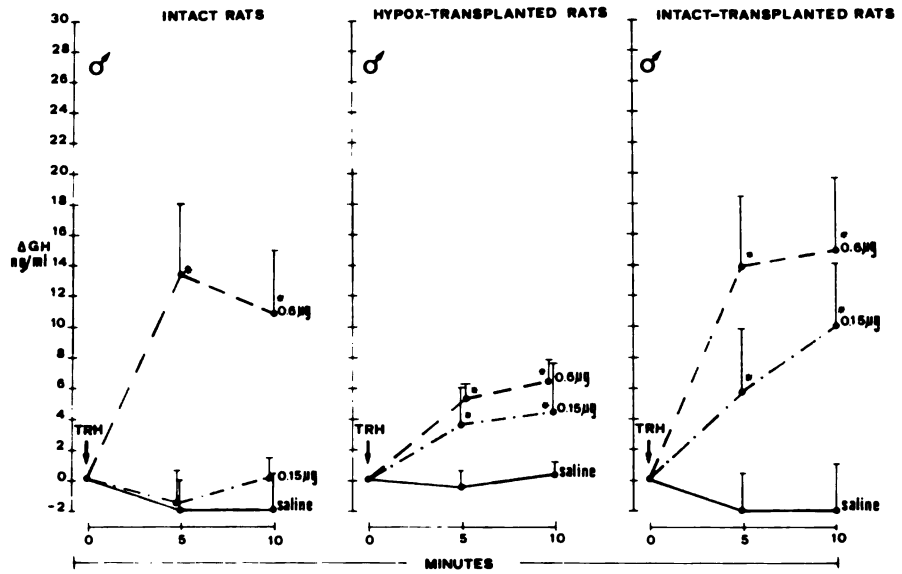

FIG. 2. GH-releasing effect of TRH in intact male rats, hypophysectomized rats with pituitary grafts and intact ; with pituitary grafts. See Fig. 1 legend for details.

e to transplantation already demon-ated in HT rats (14). Briefly, the area of crosis, which appears in the central por-n of the transplanted pituitary in the im-ediate posttransplantation period, had en invaded by mesenchimal cells. In the ne bordering the necrotic area, all cell oes though somewhat modified were still sily recognizable. Somatotrophs, al-ough containing predominantly small se-etion granules, were still abundant. Ad-nistration of TRH at the highest dose (1.2 ;) was capable of eliciting exocytosis both om the *in situ* (Fig. 3A) and the ectopic ig. 3B) pituitary.

Quantitative studies (Table I) demon-ated that in IT female rats, TRH stimu-ed exocytosis from the ectopic pituitary at doses used (0.15, 0.6 and 1.2 μg), ereas only the highest TRH dose (1.2 ;) was capable of increasing the frequency exocytosis from the pituitary *in situ*. The tent of exocytosis from the ectopic pitui-y was significantly higher than that occur-g from the pituitary *in situ* with the 0.15-d 1.2-μg doses. Because of large varia-n and the small number of animals exam-ed, the effect of the 0.6-μg dose was not tistically significant.

Discussion. In agreement with previous reports by our group (8, 9), experiments performed in this study point out the preferential release of GH by TRH from an anterior pituitary devoid of CNS influences. A relatively small rise in plasma GH induced by TRH in HT rats agrees with previous findings (8, 15) and is likely attributable to the presence of a small GH pool in the ectopic pituitary (14, 15). TRH at the doses of 0.6 and 0.15 μg in female and male rats, respectively, induced GH release in animals bearing an ectopic pituitary, irrespective of the presence of the pituitary *in situ*. TRH at the same doses was completely ineffective in intact rats and only with the highest TRH dose (1.2 and 0.6 μg in female and male rats, respectively) was a GH release elicitable. The more consistent rise in plasma GH after the highest TRH dose present in IT female rats indicates that in this instance both the ectopic and *in situ* pituitary glands were contributing to the GH increase. This pattern was less evident in the IT male rats.

Results of EM studies corroborated these findings by showing that exocytosis from the somatotrophs of the ectopic AP was highly stimulated after all TRH doses whereas only with the highest dose was exocytosis increased also in the somatotrophs of the pituitary *in situ*.

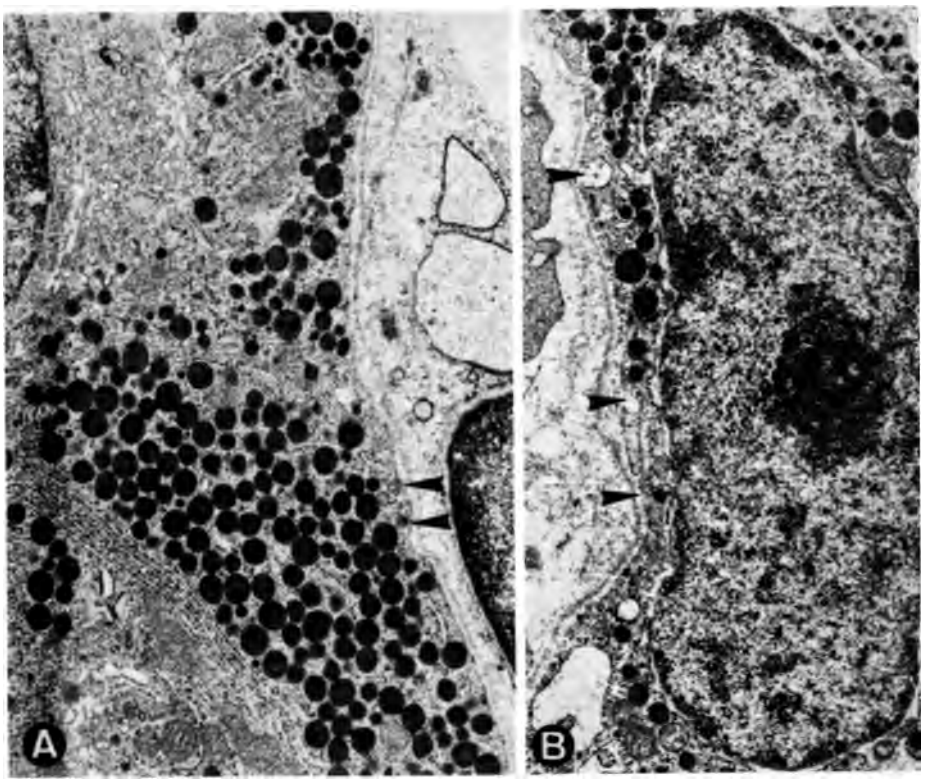

FIG. 3. Ultrastructural aspects of somatotrophs of the *in situ* (A) and ectopic (B) pituitary after 1.2 μg of TRH, showing occurrence of exocytosis (▲).

TABLE 1. EFFECT OF TRH ON THE OCCURRENCE OF EXOCYTOSIS IN SOMATOTROPHS OF INTACT RATS BEARING AN ECTOPIC PITUITARY.

Treatment	Number of rats	Exocytosis/somatotroph (mean ± SE)	
		In situ pituitary	Ectopic pitu
Saline	2	1.95 ± 0.15	2.25 ± 0.2
TRH, 0.15 μg	2	2.20 ± 0.10	4.08 ± 0.2
TRH, 0.6 μg	2	2.50 ± 0.90	9.90 ± 3.9
TRH, 1.2 μg	2	5.45 ± 0.25*	16.05 ± 1.5

* $P < 0.05$ vs saline control (ANOVAR).
** $P < 0.05$ vs *in situ* pituitary (ANOVAR).

The observation that TRH releases GH preferentially from an ectopic gland also when the latter is transplanted into an intact recipient seems to rule out the possibility that an impaired secretion of target gland hormones, e.g., gonadal steroids, thyroid hormones etc., with ensuing sensitization of the pituitary gland to the action of the tripeptide, may account for the high susceptibility to TRH of the HT rat. An intervention of "peripheral" hormones, e.g., estrogens, was made likely by the observation that the intact male is more susce the intact female rat to TRH-in release (13, 16, and this study) a GH rise or blunting of the GH re present in intact male rats given l of estrogens after systemic (17) o tal vessel (6) administration of exaggerated GH response to TI was inhibited by thyroxine pre has been reported in rats made h (18). Similarly hypothyroid hun respond to TRH with a GH ris

se was abolished following thyroid ther-
19).
spite of these findings, the higher sus-
bility to TRH of the ectopic pituitary
l also when placed in a physiologic en-
ne milieu indicates that target gland
ones do not play a crucial role except
e extremes of the secretory spectrum,
when present in supraphysiologic
nts or when totally absent.
more sound reason for the preferential
se of GH from the somatotrophs of an
ic pituitary after TRH seems to be the
nce of a CNS–anterior pituitary dis-
ection per se. Supporting this view is
bservation that a preferential release of
after TRH is also present in rats with
sive hypothalamic lesions (9) and is
-cut in the infant rat in which CNS–
ior pituitary links have been weakened
estruction of catecholamine nerve ter-
ls in the hypothalamus by 6-hydroxy-
mine (20).

mmary. Thyrotropin-releasing hor-
(TRH) was administered iv using ure-
anesthesia to either intact female and
controls or intact (IT) or hypophysec-
zed (HT) rats of both sexes bearing an
ior pituitary (AP) graft under the kid-
capsule. TRH at all the doses used
, 0.6, and 1.2 μg) elicited a clear GH
n both IT and HT rats, while only at the
st does (1.2 μg) was it effective in
t controls. Qualitative and quantitative
ron microscopic studies performed on
n situ and ectopic pituitaries from IT
le rats demonstrated that TRH stimu-
exocytosis from the ectopic pituitary at
loses used, whereas only the highest
dose was capable of increasing the
ency of exocytosis from the pituitary *in*
The similar responsiveness to TRH of
ctopic pituitary irrespective of the pres-
of an *in situ* AP, rules out the possibil-
at peripheral hormonal factors (e.g.,
of estrogens or thyroid hormones) may
a crucial role in the preferential GH
nse to TRH present in HT rats.

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Received November 18, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 578-581 (1977)

Cyproheptadine Blockade of a Cardiogenic Hypertensive Chemoreflex (39

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Introduction. Certain cardiac chemoreceptors are sensitive to serotonin (1) and their activation elicits immediate and complex reflexogenic responses within the autonomic nervous system. Components of this cardiogenic reflex include acute arterial hypertension, changes in cardiac contractile state, and profound chronotropic and dromotropic actions. Using a technique of selective perfusion of the sinus node artery and AV (atrioventricular) node artery with autonomic blocking drugs, it has been possible to analyze the multiple coexisting cardiac components of the reflex (1). Various drugs have been shown to be serotonin antagonists (2). It was the purpose of this study to determine whether some of those agents had an effect on the cardiogenic hypertensive chemoreflex.

Methods and materials. Sixteen adult mongrel dogs of either sex weighing 15–22 kg were studied. Twelve were anesthetized with sodium pentobarbital (30 mg/kg iv) and four were anesthetized with a combination of phencyclidine hydrochloride (2 mg/kg im) and α-chloralose (80 mg/kg iv). Ventilation with room air was maintained through a cuffed endotracheal tube with an intermittent positive pressure Harvard pump. The heart was exposed and the stellate ganglia were isolated by a bilateral thoracotomy. A lead-II ECG was recorded along with electrograms from bipolar electrodes sutured in the vicinity of the sinus node or left atrial appendage as well as from the midsurface of the right ventricular outflow tract or left ventricular free wall. The sinus node electrogram was used to t tachograph and central aortic bloc sure was routinely recorded. Atrial a tricular contractile forces were r with Walton–Brodie strain gauge ar tured onto either right or left atrial age or a ventricular-free wall. All were amplified and recorded on a I Packard polygraph.

The cardiogenic hypertensive c flex was induced by injection of s (50–200 μg/ml; 2 ml) into the lef through a catheter advanced via a nary vein. This amount of serotoni istered in this manner has been sho sistently to elicit the cardiogenic re Cyproheptadine hydrochloride, m gide maleate, and morphine sulfate dissolved in Ringers solution for nous administration. When parasym blockade was required, atropine mg/kg was administered intravenous dogs). Right stellate ganglion stir was performed before and after adr tion of the serotonin antagonists, their possible effect on sympathetic responses. Stimulation parameters Hz, 2 msec duration, supramaximal

Results. A. Components of the h sive cardiogenic chemoreflex. Inje serotonin into the left atrium of an tized dog induces an immediate p increase in systemic blood pressur ring within a few seconds after injection. A simultaneous vagally i sinus bradycardia can be readily bl atropine selectively perfused into node artery (1) or by bilateral v which breaks the afferent (and neural routes of the reflex. This ref bradycardia is both more rapid in o of greater magnitude than the dir tive chronotropic action of seroton sinus node (4). Concomitant with t cardia, there is an immediate va

[1] This work was supported by the National Heart and Lung Institute (Program Project Grant HL 11,310 and SCOR on Ischemic Heart Disease No. 1 P17 HL 17,667) and by the Rast Fund for Medical Research.

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diated decrease in atrial contractile force which is not rate dependent since it is still readily demonstrable during cardiac pacing. These control responses were all similar and were observed in each of the animals studied.

Figure 1 illustrates the effect of systemic atropine on the cardiogenic hypertensive chemoreflex (left panel). There is an increase in aortic blood pressure, heart rate, and contractile force in response to serotonin administration. In four dogs pretreated with atropine and utilized in the cyproheptadine studies, systolic aortic pressure increased 87 ± 14 (SEM) mm Hg, and the diastolic pressure increased 64 ± 9 mm Hg. Simultaneously there was a prompt sinus tachycardia which averaged 40 ± 9 bpm. This positive chronotropic effect is normally obscured unless muscarinic blockade is present (1). The increased atrial force is also normally obscured unless local effect of acetylcholine is blocked with atropine. The positive inotropic effect is independent of heart rate changes (observed in paced preparations) and is prevented by β-receptor blockade with propranolol (0.5–1.0 mg/kg iv).

B. Effects of cyproheptadine on the hypertensive cardiogenic chemoreflex. Intravenous administration of cyproheptadine (0.25 mg/kg) attenuates the chemoreflex. Increasing the cyproheptadine dose to 1.0 mg/kg (Fig. 1, right panel) abolished the cardiogenic chemoreflex in 9 of 10 dogs. In these dogs serotonin produced only minimal or insignificant changes in blood pressure, heart rate, or contractile force. The degree of blockade of the serotonin reflex wanes with time, but is still partially apparent for up to 2 hr.

C. Sympathetic neural effects before and after cyproheptadine. Possible β-receptor blocking actions of cyproheptadine (known to have a minor atropine-like property (5)) were tested by right stellate ganglion stimulation before and after cyproheptadine 1.0 mg.kg iv. Stellate stimulation induced the same positive chronotropic and inotropic effects before or after cyproheptadine in each of six dogs so tested (Fig. 2).

D. Surmounting the cyproheptadine block. Although cyproheptadine (1 mg/kg) effectively blocked the maximal response of the chemoreflex when 100 μg/ml of serotonin was used (2 ml), this blocking effect could be overcome in each of the three dogs so tested by a 10-fold increase in the serotonin concentration (Fig. 3). Bilateral cervical vagotomy, however, abolished the hypertensive chemoreflex elicited by 1000 μg/ml of serotonin (2 ml). Such "reversal" of the cyproheptadine blockade suggests that serotonin and cyproheptadine may compete for receptor sites in initiation of the reflex.

E. Lack of effects of methysergide and morphine on the hypertensive cardiogenic chemoreflex. Intravenous administration of

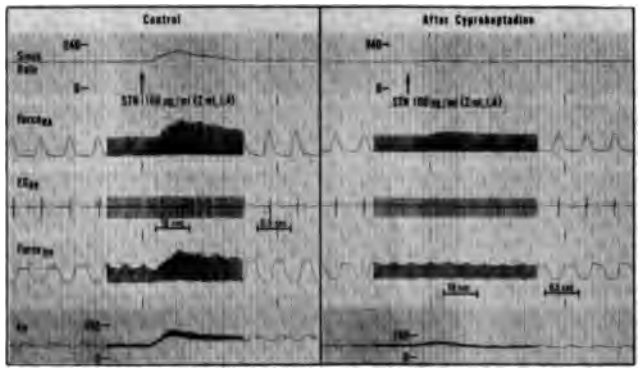

FIG. 1. *Left*: A characteristic example in an anesthetized, atropinized dog of the cardiogenic hypertensive chemoreflex elicited by administration of serotonin. $Force_{RA}$, right atrial contractile force from a Walton-Brodie strain gauge; EG_{RV}, right ventricular electrogram; $Force_{RV}$, right ventricular (conus) contractile force with a Walton-Brodie strain gauge; Ao, central aortic pressure. *Right*: Cyproheptadine (1.0 mg/kg iv) has virtually abolished the cardiogenic hypertensive chemoreflex.

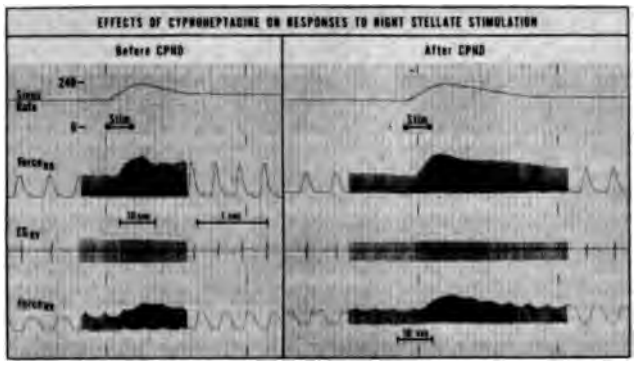

FIG. 2. Comparison of the cardiovascular effects of right stellate ganglion stimulation before (left) and after (right) 1.0 mg/kg of cyproheptadine (CPHD).

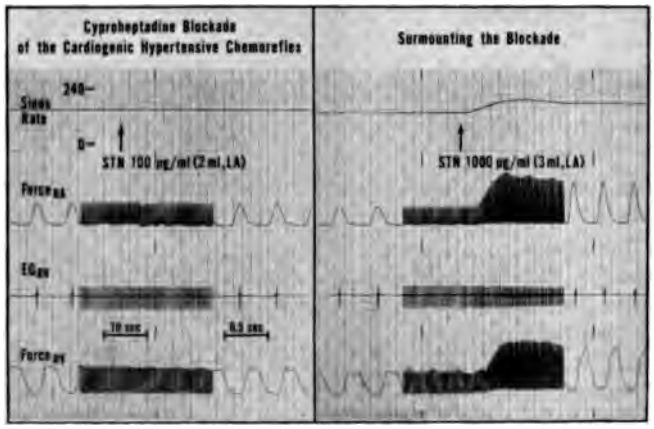

FIG. 3. Cyproheptadine blockade of the chemoreflex (left panel) can be surmounted with a 10-fold increase in the concentration of serotonin (right panel). The original response to serotonin in the standard test concentration (100 µg/ml) is shown for this same dog in Fig. 1.

methylsergide, 10 to 100 µg/kg, failed to block or attenuate the serotonin induced cardiogenic reflex in five of seven dogs. Increasing the methysergide concentration to 1–2 mg/kg in two dogs also failed to affect the chemoreflex but produced hypotension. This hypotension may be attributable to the ergot alkaloid nature of methysergide (2).

Intravenous morphine sulfate [known to block serotonin in some preparations (6)] was administered in increments of 500 µg/kg doses in three dogs. Even at a total concentration of 2 to 3 mg/kg morphine failed to prevent the serotonin induced cardiogenic hypertensive chemoreflex.

Discussion. Cyproheptadine (but not methysergide or morphine) effectively blocks all components of a hypertensive cardiogenic chemoreflex regularly elicitable with serotonin. The blockade appears to be competitive in nature and at the site of initiation of the reflex since it can readily be surmounted by an appropriate increase in the concentration of serotonin. Because the cyproheptadine blockade can be overcome with greater concentration of serotonin, it is unlikely that central nervous system depression by cyproheptadine played an important role in the blockade of the serotonin reflex. Furthermore the results indicate cyproheptadine has no significant antiadrenergic nor β-receptor blocking effects.

Any human counterparts to this experimental hypertensive cardiogenic chemoreflex remain to be defined, although the histological similarity of chemoreceptor tissue near the main left coronary artery of both human and canine hearts supports the possi-

bility of a similar reflex in man. Clinical circumstances in which this chemoreflex may participate include the severe hypertension sometimes observed in patients with angina pectoris or acute myocardial infarction. If further studies support this speculation, clinical trial of cyproheptadine to treat such hypertensive crises merits consideration.

Summary. A cardiac chemoreceptor can be activated by serotonin to cause a complex autonomic reflex which includes acute hypertension, changes in cardiac contractile force, and alteration of rhythm and conduction. In 16 anesthetized dogs blood pressure, heart rate, and contractile force (Walton-Brodie strain gauges sutured on atria and ventricles) were continuously measured during serotonin-induced reflexes before and after intravenous administration of cyproheptadine and/or methysergide or morphine. Cyproheptadine in concentrations of 0.2–0.5 mg/kg attenuated the reflex responses, and higher concentrations (1.0 mg/kg) abolished the reflex (9 of 10 dogs). The responses to right stellate ganglion stimulations before and after cyproheptadine were identical, indicating that cyproheptadine had no effect on neural release of norepinephrine nor any significant β-receptor blocking action. Cyproheptadine blockade was readily surmounted by a 10-fold increase in serotonin concentration. The serotonin reflex progressively recovered in time after the administration of cyproheptadine. Central nervous system depression accounting for this abolishing effect of cyproheptadine is unlikely because of the surmounting effects of larger concentrations of serotonin. Methysergide and morphine had no significant effect in preventing the serotonin reflex. Cyproheptadine most likely interferes with the effect of serotonin at the site of initiation of the reflex within the cardiac chemoreceptor.

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Received November 11, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 582-586 (1977)

Vasa Vasorum within the Media of Bovine Mesenteric Lymphatics (3

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In lower animals, particularly amphibia, the flow of lymph is maintained by rhythmically beating lymph hearts. Although there are no lymph hearts in the mammal, all lymphatics other than terminal capillary networks contain smooth muscles in their wall (1). It has been noted that mammalian lymphatics, at least in certain species and in certain areas, showed spontaneous active contractions which were capable of promoting lymph propulsion (1). Recently, the present authors have succeeded in recording membrane action potentials from bovine mesenteric lymphatics simultaneously with phasic contraction waves which had one-to-one correspondence to the action potentials. The present electron microscopic study was undertaken to elucidate the motor activity of lymphatic smooth muscles from the structural point of view. It was found that blood capillaries are distributed within and among the smooth muscle layers.

Materials and methods. Segments of mesenteric lymphatics, 2–3 cm in length, were dissected from the fresh mesenterics of recently slaughtered cattle and fixed in a 4% glutaraldehyde solution. Small pieces were then cut for embedding. After fixation in osmium tetroxide and dehydration in alcohol, the tissue blocks were embedded in epon. All sections were cut with the LKB Ultrotome, and the electron microscopy was done with the Hitachi MU-11A. The sections were stained double with lead citrate and uranyl acetate.

Results and discussion. Figure 1 is a transverse section of bovine mesenteric lymphatics at magnifications of 700 diameters to demonstrate the general organization of the lymphatic wall. Smooth muscles, which lined the walls very thickly, could be distinguished in three layers, i.e., the internal longitudinal, intermediate circumferential, and external longitudinal. The outermost layer was much thicker than the other two. There existed few elastic fibers and a vast number of collagen fibers unde endothelial lining and among t muscle layers. Such a triplicate of lymphatic smooth muscles has noticed except in an observatio waki on human leg lymphatics (

In addition, blood capillaries not only in the adventitia but also among the smooth muscle layers rowheads in the figure indicate th at which blood capillaries were f were identified as blood capilla presence of basement membra configuration of endothelium (F casionally erythrocytes were als the lumen.

The walls of lymphatic vessels known to be supplied by a rich nutritive blood vessels located ex the adventitia (1). The present e croscopic observation revealed capillaries, along with collagen tered deep into the well-develo muscle layers of bovine mesente ics. The distribution of blood c lymphatic walls may be much spread than has so far been be presence of vasa vasorum withir may reflect the relatively high quirements of the lymphatic smo and the relatively low oxygen s the lymph flowing through the vessel. An ample supply of oxy required to maintain rhythmic c which act as a driving force for sion of lymph.

The oxygen requirements smooth muscles can be supplie blood in the lumen until the w too thick to be nourished withou rum. The critical thickness, that fusion from the vessel's own lu cient, has been estimated to be t and 1.0 mm for human aortic Wolinksy and Glagov (4) foun vasorum within the media were

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FIG. 1. Transverse section of bovine mesenteric lymphatic wall ×700. The top is the endothelial side. V
veloped smooth muscles are arranged in three layers, i.e., the internal longitudinal, intermediate circumfe
l, and external longitudinal. The outermost layer is much thicker than the other two. A large number of coll
ers exist underneath the endothelial lining and among the smooth muscle layers. Blood capillaries are fou
e locations indicated by arrowheads.

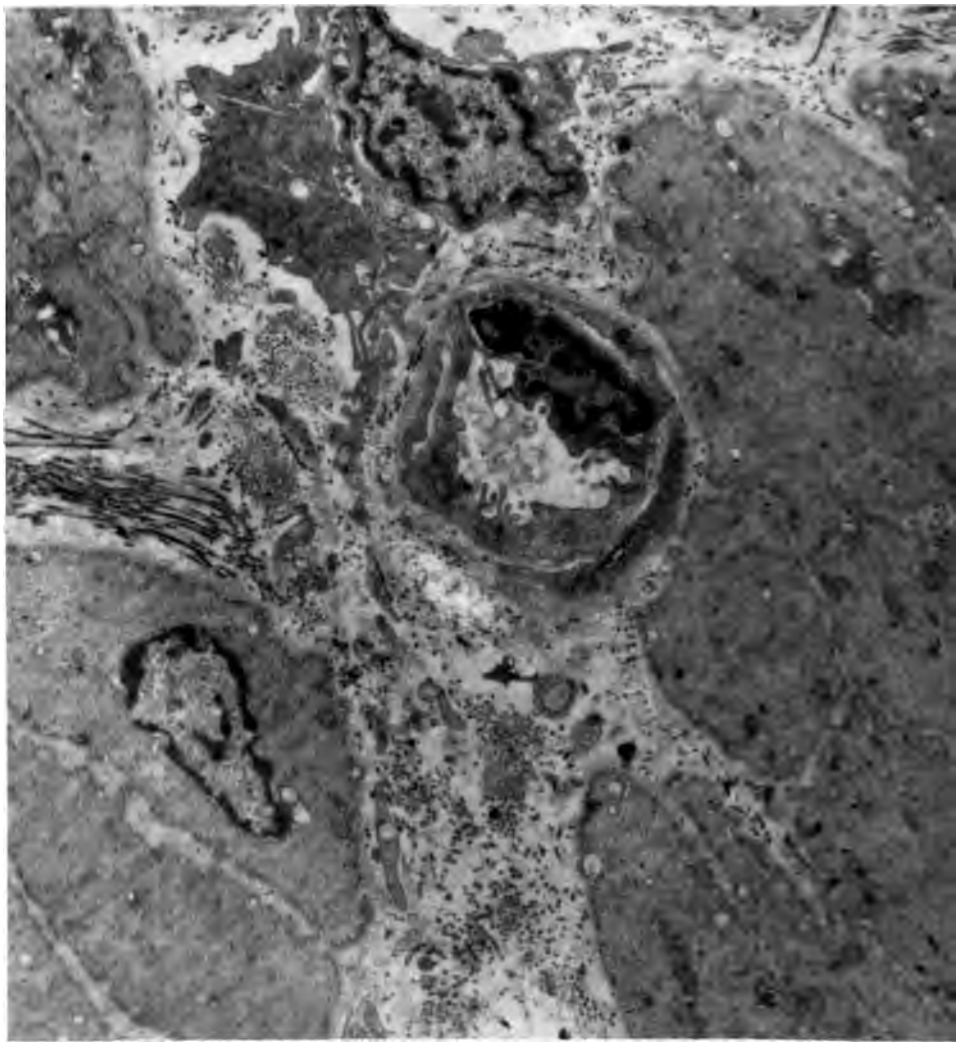

FIG. 2. Transverse section of a blood capillary found within the external longitudinal smooth m ×1540. The endothelium has many pedicles which protrude into the lumen.

mammalian aortae which had more than 29 elastic laminae but not in those which had less elastic laminae.

The amount of oxygen in lymph and the rate of flow in lymphatics are extremely low compared with those in arterial blood and in arteries (5). Hence, it may be comprehensible from the teleological point of view that the presence of blood capillaries within the well-developed smooth muscle layers is essential for bovine mesenteric lymphatics to maintain vigorous spontaneous activity. We have also found blood capillaries within the smooth muscle layers of the canine ureter and portal vein which were know spontaneous rhythmic contracti blood capillary has been found in t of bovine mesenteric arteries.

Figure 3 demonstrates a longitu tion of a lymphatic smooth muscl large number of mitochondria gath cluster were seen on both sides o cleus along the longitudinal cell merous glycogen granules were al among and around the mitochondi bers of micropinocytotic vesicle have recently been recognized as t vation site of free calcium ions

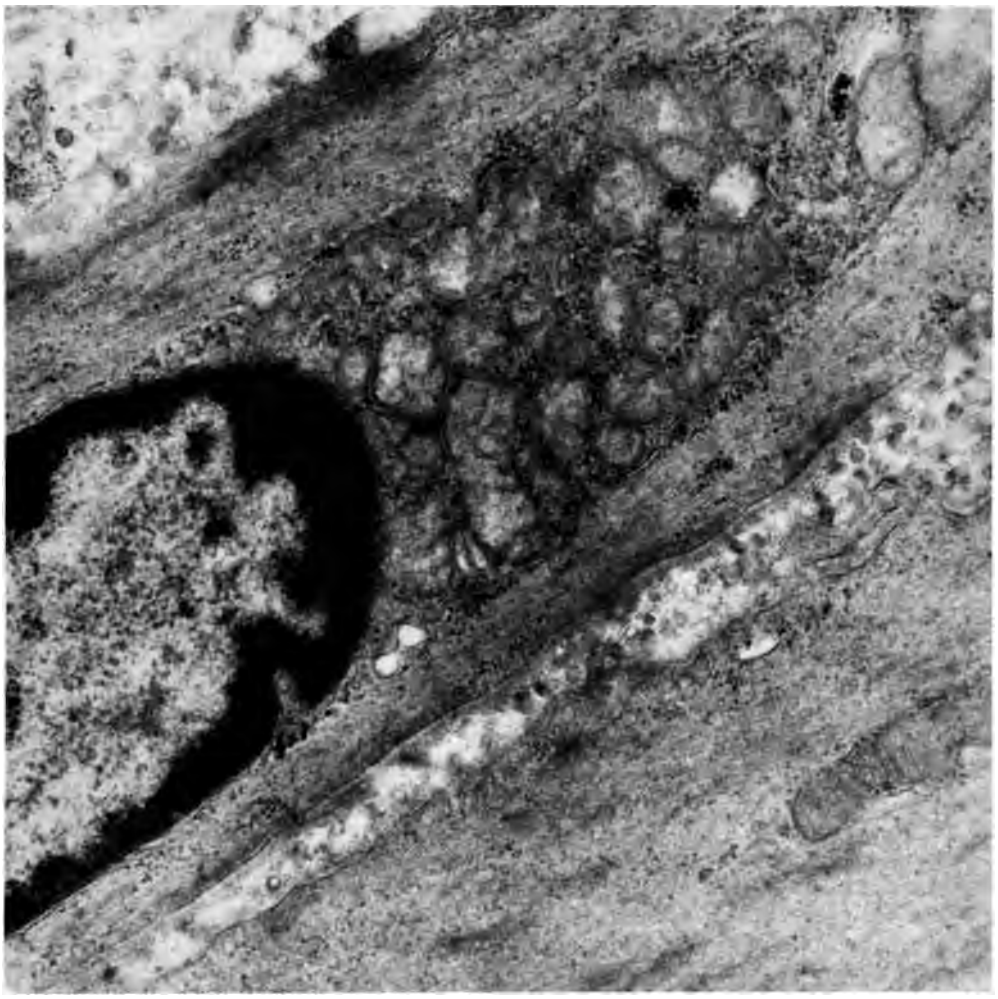

FIG. 3. Longitudinal section of a lymphatic smooth muscle cell ×7000. Mitochondria are clustered on one end of the nucleus along the longitudinal cell axis. A number of glycogen granules are seen among and around the mitochondria.

found together with mitochondria and glycogen granules in the vicinity of sarcolemma, not shown clearly in this figure. These structural features might be a morphological manifestation of the high metabolic activity required for spontaneous contractions of bovine mesenteric lymphatic smooth muscles. The high metabolic demand may be met by oxygen supply from the blood flowing through the blood capillaries distributed within the smooth muscle layers.

In the case of bovine mesenteric arteries and veins, only a few mitochondria were noticed around the nucleus of smooth muscle cells. No significant difference in other organellae was noted between lymphatic and vascular smooth muscle.

Summary. Smooth muscles in bovine mesenteric lymphatics were well-developed and arranged in three layers, i.e., the internal longitudinal, intermediate circumferential, and external longitudinal. The outermost one was much thicker than the other two. Blood capillaries were found within the smooth muscle layers as well as in the adventitia. These blood capillaries may be essential for maintaining vigorous rhythmic contractions of the lymphatic smooth muscles which act as a driving force for the

propulsion of lymph. The presence of numerous mitochondria and glycogen granules seemed to reflect a high metabolic activity of the smooth muscle cells.

The authors are indebted to Dr. T. Suganuma and Mr. R. Ichikawa for their technical advice and assistance.

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Received October 20, 1976. P.S.E.B.M. 1977, Vol. 154.

EDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 587-590 (1977)

Tolerance to the Effect of Morphine on Intestinal Transit[1] (39724)

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ıtroduction. The repeated or "chronic" ction of a narcotic analgesic, exempli- by morphine, often results in the phe- ıena of tolerance and physical depend- e (1-3). In regard to the gastrointestinal t, however, the responses to repeated ıinistration of morphine are not clear. erance to the effects of morphine on in- inal contractions *in vitro* appears to de-)p. The isolated ileum of the guinea pig ıhibited by morphine and tolerance de-)ps to this inhibition (4). Also, tolerance elops to morphine's stimulation of dog ated small intestine (5). On the other d, results on the development of toler- e to the intestinal actions of morphine *in*) are meager and conflicting. Miller and nt monitored intraluminal pressures n the intestines of unanesthetized dogs found that tolerance did not develop to stimulatory effects of morphine (6). In trast, Thor *et al.* monitored the myoelec- activity of the small bowel of the unan- ıetized dog and found that tolerance did elop to the stimulant action of morphine

Motility functions of the small intestine be investigated by following the transit a nonabsorbable marker. Alterations in ısit are believed to be secondary to nges in the contractile patterns of the ıll intestinal musculature. This study was designed to: (i) assess quantitatively whether acute injections of morphine affect intestinal transit in unanesthetized rats, and (ii) determine whether prior treatment of the rats with repeated injections of morphine produces tolerance to the observed effects.

Materials and methods. Intraduodenal catheters were implanted in 32 Sprague-Dawley rats (Sprague-Dawley, Madison, Wis.) weighing 203 ± 4 g (mean ± SE). Details of implantation have been reported (8). Briefly, the animals were anesthetized with 20 mg of ketamine hydrochloride (Ketaject, Bristol Laboratories, Syracuse, N.Y.) administered intraperitoneally. A tygon catheter, 0.51-mm inside diameter and 1.51-mm outside diameter, was implanted into the duodenum through a small incision made at the anterior surface of the greater curvature of the stomach. The other end of the catheter was passed between the skin and musculature at the midline incision to exit through a small cutaneous puncture made in the midscapular region. The abdominal incision was closed in two layers. The exposed end of the catheter was sealed with collodion and taped to the back of the neck.

The animals were allowed to recover for 3 to 5 days (all animals were eating at this time and were gaining weight). They were then divided into three groups. Members of group 1 (naive-saline) were injected subcutaneously with saline and intestinal transit was determined the same day. Rats in group 2 (naive-morphine) were injected with saline three times daily for 7 days and intestinal transit was determined on the 7th day. Rats in group 3 (morphine treated-morphine) were injected with morphine sulfate, 20 mg/kg, three times daily (8:00 AM, 4:00 PM, and midnight) for 7 days and intestinal transit was determined on the 7th day. All

This study was supported in part by USPH Grants 00801 and AI 15361. Dr. Castro is the recipient of Research Career Development Award AI 00087. Weisbrodt is the recipient of USPHS Research ntist Development Award DA-00022.

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ISSN 0037-9727

injections were given subcutaneously.

On the day that intestinal transit was determined, each rat was injected subcutaneously with either saline or morphine sulfate, 20 mg/kg. In the case of group 3 animals, the morphine injection came at either 8:00 AM or 4:00 PM so that the established pattern of injections was maintained. Determination of intestinal transit was then initiated either 30, 60, or 120 min after injection of either saline (group 1) or morphine (groups 2 and 3). Intestinal transit was determined using a modification of the technique described by Summers *et al.* (9). The tape was removed from the backs of the rats and the catheter was opened by cutting the tip previously sealed with collodion. The catheter was cleared with 0.05 ml of 0.9% saline, followed by subsequent instillation of 0.2 ml of saline which contained approximately 0.5 μCi of $Na_2{}^{51}CrO_4$ (hereafter referred to as ^{51}Cr). Intraduodenal instillation of ^{51}Cr solution was followed immediately by flushing with 0.05 ml of saline. The volume of the saline flush was greater than the void volume of the catheter and was volume sufficient to wash out consistently more than 99% of the previously injected isotope. All animals were killed by cervical dislocation 25 min after instillation of the ^{51}Cr. Two milliliters of blood were immediately obtained by heart puncture and then the abdomen was opened. Silk was used to tie the small intestine at the tip of the catheter and at the ileocecal junction. Other ties were made at the junction of the cecum and colon, and at the pylorus while withdrawing the catheter from the stomach. The GI tract was cut at the point of the ties and carefully removed by cutting away the mesentery.

Once removed, the intestine was divided by metal clips into 10 equal segments of approximately 10 cm each beginning at the tip of the catheter and proceeding distally. The intestine was cut at these clips. Each segment, as well as the cecum and the blood samples, were placed in vials. The distribution of ^{51}Cr in the blood and throughout the small bowel and cecum was assayed by monitoring the radioactivity in each vial with a Searle Model 1195 γ-counter. Data were expressed as percentage of distribution of radioactivity present or traversing a given segment of intestine during the 25-i period. The amount of radioacti covered from the 10 intestinal segm considered 100%.

Results. Neither placement of the odenal catheter nor the daily inje morphine sulfate interfered with mals' ability to eat and gain weig naive rats gained 18.4 ± 6.9 g durin of saline injections. The morphine rats gained 24.8 ± 5.4 g during t period. During the 1st day of morp ministration, the animals appeared a catatonic state. After the first 2- however, their behavior appeared i to that of the naive rats.

All blood samples were free of rad ity, thus the ^{51}Cr was not absorbed f gut. Measurement of gut transit strated that in the naive rats treat saline (group 1), chromium (25 m instillation) was distributed along th mal 90% of the gut length (Fig. 1) administration of morphine to nai (group 2) caused a marked change

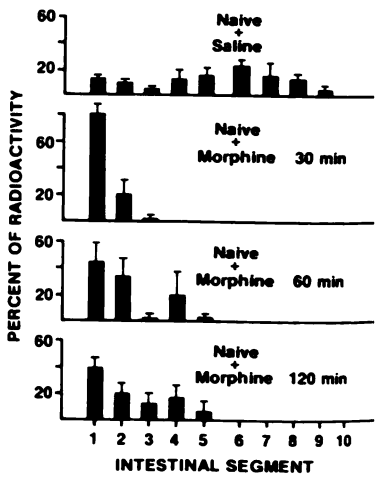

FIG. 1. Distribution of radioactivity alo length of the intestine 25 min after instillation into the duodenum. Naive rats received salin days before testing. Time refers to the interval b injection of morphine and instillation of ^{51}Cr segment corresponds to 0.1 of the total length intestine. Segment 1 contains part of the duc and segment 10 contains the terminal ileum. and standard errors are for 7, 4, 4, and 5 rats un conditions of saline, and morphine at 30, 60, a min, respectively.

distribution of ^{51}Cr in the GI tract. When ^{51}Cr was instilled 30 min after morphine administration, it never traversed more than 30% of the gut length. If the duration between morphine injection and ^{51}Cr instillation was 60 or 120 min, the chromium traversed 50% of the gut length.

Acute administration of morphine sulfate, 20 mg/kg, to the morphine-treated rats (group 3) also caused a decrease in transit of ^{51}Cr (Fig. 2). The decrease, however, was less than that produced in the rats comprising group 2. The ^{51}Cr front in rats tested 30, 60, and 120 min after the last injection of morphine traversed 70, 70, and 80% of the gut, respectively.

The means of the segments reached by the ^{51}Cr front are shown in Fig. 3. The front of the chromium traversed the shortest distance in the naive animals that were injected with morphine (group 2). The difference in levels reached was significant in the naive animals given saline (group 1) as compared to the naive rats given morphine (group 2) regardless of the interval of time (30–120 min) between morphine injection and testing. The distance traversed after morphine injection was also significantly less in the naive animals (group 2) compared to the morphine-treated animals (Group 3) at each respective time interval. There was no significant difference in the level reached in the morphine-treated rats that received morphine and were tested at 120 min compared to the naive rats that were injected with saline.

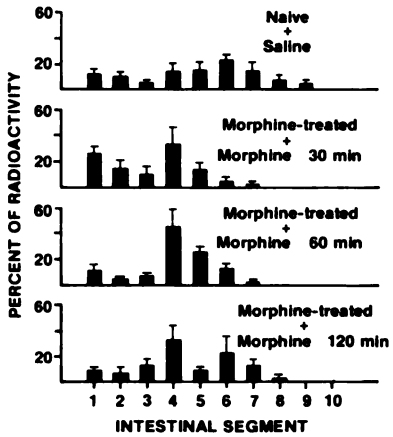

FIG. 2. Distribution of radioactivity along the length of the intestine 25 min after instillation of ^{51}Cr into the duodenum. Naive rats received saline and morphine-treated rats received morphine for 7 days before testing. Time refers to the interval between injection of morphine and instillation of ^{51}Cr. Each segment corresponds to 0.1 of the total length of small intestine. Segment 1 contains part of the duodenum and segment 10 contains the terminal ileum. Means and standard errors are for 7, 4, 4, and 4 rats under the conditions of saline, and morphine at 30, 60, and 120 min, respectively.

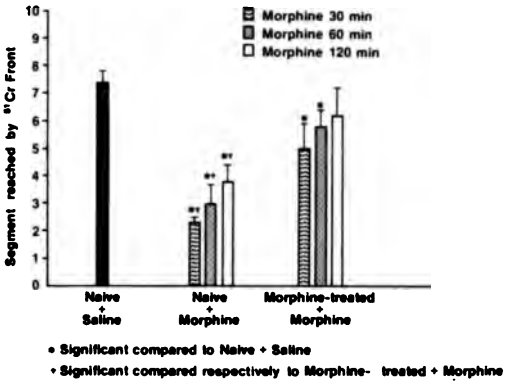

FIG. 3. The most distal segment reached by the ^{51}Cr 25 min after instillation into the duodenum. Naive rats received saline and morphine-treated rats received morphine for 7 days prior to testing. On the test day, either saline or morphine was injected 30, 60, or 120 min before instillation of the ^{51}Cr. A difference was considered significant if $P < 0.05$.

The mean of the most distal segments traversed by 50% of the chromium also showed differences among the three groups (Fig. 4). The distance was shortest in the naive rats that received morphine (group 2). The differences in distances between the naive rats given saline (group 1) and those given morphine (group 2) were significant at all three time intervals tested. At each respective time interval, the difference in distal traversed by 50% of the ^{51}Cr in the naive rats given morphine (group 2) was also significantly different from the distances traversed in the morphine-treated rats given morphine (group 3). There were no significant differences between the naive rats given saline (group 1) and the morphine-treated rats given morphine (group 3) regardless of the time interval.

Discussion. The techniques utilized in this investigation have several advantages. Two of the most important advantages are that

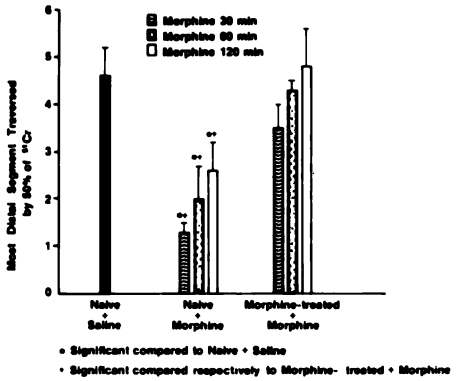
Morphine 30 min
Morphine 60 min
Morphine 120 min
Most Distal Segment Traversed by 50% of 51Cr
Naive + Saline
Naive + Morphine
Morphine-treated + Morphine
* Significant compared to Naive + Saline
** Significant compared respectively to Morphine- treated + Morphine

FIG. 4. The most distal segment traversed by 50% of the ^{51}Cr 25 min after its instillation into the duodenum. Naive rats received saline and morphine-treated rats received morphine for 7 days prior to testing. On the test day, either saline or morphine was injected 30, 60, or 120 min before instillation of the ^{51}Cr. A difference was considered significant if $P < 0.05$.

the animals are unanesthetized during determinations of intestinal transit and that the marker is administered directly into the small intestine, thus avoiding the variable of gastric emptying. Disadvantages of the technique are that we are following the movement of material for a relatively short time and that measurements of intestinal transit alone give little insight into the mechanisms by which transit may be altered.

Our results on transit in naive rats given saline and morphine compare well with those reported by others (8, 9). In addition, we found that the magnitude of the decrease in transit produced by morphine depended on the interval of time between the injection of morphine and testing. While transit was decreased when tested 120 min after morphine injection, the decrease was much less than when tested 30 min after morphine injection. The attenuative effects of morphine with time may be attributed to decreasing plasma concentrations since plasma levels of morphine are known to fall dramatically during the 2-hr period after injection (10).

The main observation we wish to s that, after repeated injections of mor the effects on intestinal transit normal duced by an acute dose of morphin markedly diminished or lost. Thes agree with the recent observation chronic morphine treatment decrea responses of dog intestine to acute d morphine *in situ* and *in vitro* (5). Our differ, however, from those of Mill Plant (6) and from the stated impression that tolerance does not d to the constipating effect of the narco 2). In regard to constipation, our must be viewed with caution since co tion could be caused by changes oth those related to transit through the intestine. Nevertheless, our study sents an *in vivo* demonstration of mo tolerance on transit through the smal tine.

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Received May 24, 1976. P.S.E.B.M. 1977, V

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 591-596 (1977)

Effect of SO_2 on the Pathogenesis of Viral Upper Respiratory Infection in Mice (39725)

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Introduction. Epidemiologic studies have consistently shown an association between respiratory infection and air pollution. Most of these studies have been concerned with effects on the mucous membranes of the tracheobronchial tree (1-5). Among the many airborne contaminants to which the Yokkaichi industrial area of Japan was heavily exposed from about 1960-1966, SO_2 was the major pollutant, with levels ranging from 0.2 to 2.5 ppm (6), and during those years the incidence of respiratory diseases was significantly increased over that of nonpolluted areas. In 1968, after rigorous control measures, the range was lowered to 0.071-1.0 ppm. Nonetheless, Ohyana and Miyoshi found in 1972 that the incidence of rhinitis and tonsillitis in school children in Yokkaichi was still significantly higher than that of children in nonpolluted areas (7). Previous reports of increased respiratory disease had been concerned with industrial workers in the United States and in England after exposure to SO_2 levels as high as 55 and 36 ppm, respectively (8).

There have been several histopathologic studies of lower respiratory mucosae in experimental animals after exposure to various levels of SO_2 (9-16) and three studies of experimental exposure of nasal mucous membranes to high (10-36 ppm) concentrations of SO_2 (17-19), but the effects of *continued* exposure of nasal tissues to *low levels* of SO_2 have not been reported. Since the inhaled gas initially impinges on nasal tissues and may interact with other factors to enhance respiratory tract infections, it seemed important to follow the pathogenesis of intranasal virus infection in association with low levels of exposure. The present study was designed to determine the effects of constant exposure of nasal mucous membranes of mice to low levels of SO_2 on the pathogenesis of influenza virus infection in these animals.

Materials and methods. Animals. Male mice of the Japanese DS strain, derived from the commercial dds strain maintained by the Abulahi Laboratory, Shionogi Co., Shiga prefecture, Japan, were used throughout the study. They were all 4-5 weeks old and weighed 15-20 g when first exposed to SO_2.

Virus. The mouse-adapted PR_8 strain of influenza A virus was serially passed six times in unanesthetized mice by intranasal inoculation. After each passage, macerated lung tissues were diluted tenfold in normal saline and used as the successive inocula. Lung tissues from the final passage were inoculated into the allantoic fluid of 12-day-old chick embryos. Allantoic fluid was harvested 24 hr after inoculation and was stored at −70° until used. The virus had a hemagglutination titer of approximately 2024.

Exposure condition. The SO_2 exposure apparatus is shown in Fig. 1. During exposure, approximately 30 mice were housed in 3290-cm^3 stainless steel wire cages within the vinyl chloride exposure chamber, which had a volume of 6880 cm^3. Sulfur dioxide was generated by a standard gas generator (Kimoto SCCI, 3780), utilizing a Teflon permeation tube containing liquified SO_2. The gas was mixed with filtered air and introduced into the exposure chamber at a rate of 9 liter/min. Sulfur dioxide was maintained at a concentration between 0.03 and 0.1 ppm throughout the exposure period. The SO_2 concentration was determined daily by the *p*-rosaniline method (20). Food and water were provided *ad libitum*.

Experimental procedure. Mice were divided into three experimental groups of five

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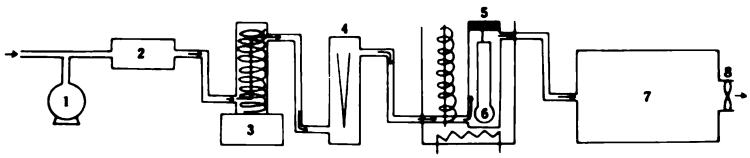


FIG. 1. Diagram of SO_2 exposure apparatus: (1) air compressor; (2) air filter containing activated charcoal and silica gel; (3) refrigerator; (4) flow meter; (5) water bath, 25°; (6) SO_2 permeation tube; (7) exposure chamber; (8) exhaust fan.

mice each: (i) controls which were exposed to neither virus nor SO_2; (ii) those receiving virus alone; and (iii) those exposed to SO_2 and then inoculated with virus. Following 4 weeks of exposure to SO_2, the mice in group 3 were inoculated intranasally with 0.02–0.03 ml of undiluted virus suspension; they were not anaesthetized. The mice were killed at 3, 6, and 12 hr, and 1, 2, 3, 5, 7, 14, and 21 days after inoculation. Mice in the two control groups were killed at the same time intervals. Body weights of mice in each experimental group were recorded through the experiment.

Histopathologic examination. After removing the lower jaw, skinned heads were fixed in 10% formalin, then decalcified in 8% trichloroacetate. Tissues were dehydrated in graded ethyl alcohol and embedded in paraffin. Transverse sections were made across the nasal cavity through the anterior ridge of the orbits, and 6–8-μm sections were stained with H&E and PAS. PAS-positive cells were counted in the respiratory epithelia of the nasal septal mucosa. All mice in all groups were evaluated histologically, and all slides were read blind.

Titration of influenza antibody in serum. Venous blood was taken from the eye. The sera were separated and inactivated at 56° for 30 min. Sera from each group of five mice were pooled, and HI antibody was titrated for each group at each time interval after inoculation. Four hemagglutinating doses of viral antigen were used in these assays.

Results. Body weight. In the virus control group, the mice lost approximately 10% of their body weight from the prechallenge level during the first 7 days after inoculation, then subsequently showed normal weight gains. The group exposed to SO_2 also lost 10% of their body weight by the 7th day after the initial exposure, and thereafter gained weight at normal rates. In the SO_2 plus virus group, the mice lost 5% of their body weight during the first 2 days after virus inoculation, and thereafter showed the same daily increments as did the virus controls.

Development of serum antibody titers. In the virus control group, HI antibody developed on Day 10 postinoculation and reached maximum levels on Day 14. In the SO_2 plus virus group, these levels reached 1024 on Day 6 and rose to 4096 by Day 8 after inoculation (Fig. 2). Thus, the antibody response was significantly more rapid; at 7 days the HI titer was 500 times greater in the SO_2-exposed group. There is no known statistical test that can be applied to these geometric mean titers.

Histopathology. At 3 hr after virus inoculation, the virus control and SO_2 plus virus groups showed no histopathologic differences.

By 12 hr, there were scattered discrete changes in the middle portion of the nasal septum in the virus control group: loss of cilia (arrow), vacuolization of the epithelial columnar cells, and inflammatory cell infiltration in the subepithelial layer (Fig. 3). At the same time the SO_2 plus virus group showed extensive loss of cilia, inflammatory cell infiltration in the mucosa, and congestion of the subepithelial capillaries (Fig. 4).

At 48 to 72 hr after inoculation, both groups showed focal desquamation of respiratory epithelia in the nasomaxillary turbinates and nasal septum, with frequent fibrinopurulent exudation into the nasal cavity. In the SO_2 plus virus group, the changes were much more extensive in both extent and severity, with complete loss of cilia, areas of flattened respiratory epithelium, many islands of desquamated respiratory epithelium, and great masses of fibrinopurulent exudate in the nasal cavity lumen. The

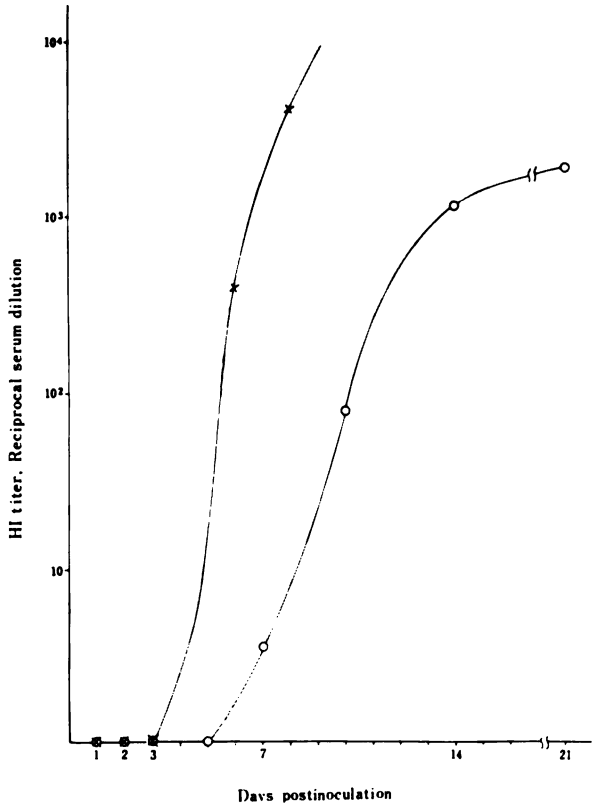


FIG. 2. Changes in HI antibody titer after intranasal inoculation of influenza virus. ×——×, SO_2 plus virus; O——O, virus only.

olfactory mucosa was also inflamed.

By 5–14 days, the virus control group showed a low columnar respiratory epithelium with few cilia, and intensive desquamation of the olfactory epithelium. In the SO_2 plus virus group, there was beginning repair of the septal and maxillary turbinate epithelia. The subepithelial layer was only loosely infiltrated by small numbers of inflammatory cells, and the olfactory epithelium was partially replaced by ciliated cells.

By 2 weeks postinoculation, the nasal mucosae were essentially restored in both groups. The sequence of pathological changes is summarized in Fig. 5. Histopathological changes peaked on Day 7 after inoculation in the virus controls and 2 days postinoculation in the SO_2 plus virus group.

Number of goblet cells. After 4 weeks of exposure to SO_2, the number of goblet cells in the respiratory epithelium of the nasal septum was found to be six times greater than in nonexposed mice. In the virus control mice, the number of goblet cells increased progressively after inoculation throughout the experiment. In mice exposed to both virus and SO_2, goblet cells increased for 3 days postinoculation and then rapidly decreased (Fig. 6).

Discussion. There is considerable literature linking increased frequency and severity of lower respiratory disease with exposure to high levels of SO_2. To select clinical examples from three communities: French *et al.* (1) found that excessive rates of acute respiratory disease were clearly linked to heavy SO_2 pollution; Buechley *et al.* (21) found mortality rates greater than expected when SO_2 levels were above 500 $\mu g/m^3$; and Yoshida *et al.* (6) reported that prevalence of bronchial asthma was directly proportional to concentrations of SO_2.

The nose has been curiously neglected in clinical and experimental studies of SO_2 ⇋

virus interactions, even though it is known that nasal mucous membranes absorb most inhaled SO_2 (22) and that both irritant gases and virus infections initiate inflammatory and immune responses in the nose. Matsumura's (23) concept that under certain conditions SO_2 may have an adjuvant effect on antibody production seems reinforced by the present experiments in which HI responses were more rapid and reached higher titers when virus was introduced after long-term exposure to SO_2.

The histopathologic changes induced by *virus alone* in the nasal mucosae of mice in the present experiments were similar to those described after intranasal inoculation of influenza virus in unanesthetized ferrets (24) and mice (25), and of NDV inoculation in chickens (26).

The increased numbers of goblet cells produced in nasal tissues by *SO_2 alone* were similar to responses described after long-

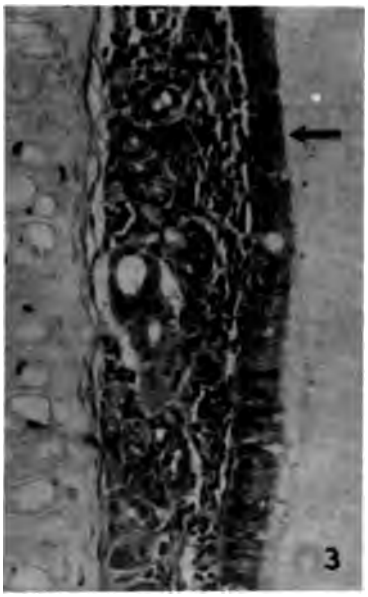


FIG. 3. Epithelium of midseptum 12 hr postinoculation, virus only, showing moderate loss of cilia (arrow) and mild inflammatory response.

FIG. 4. Epithelium of inferior turbinate 12 hr post inoculation after SO_2 exposure showing epithelial cel metaplasia and intensive inflammatory response.

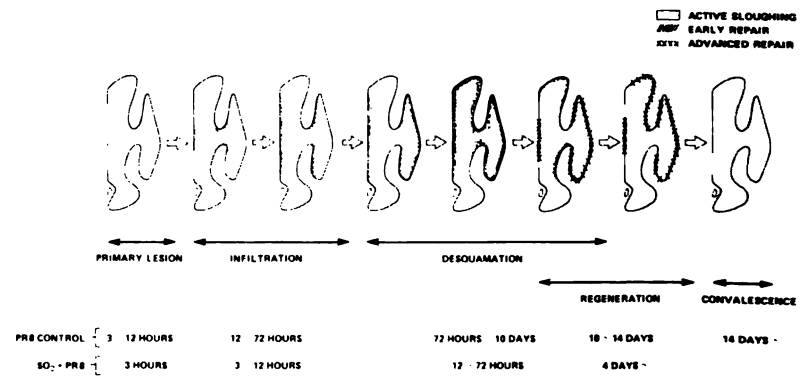
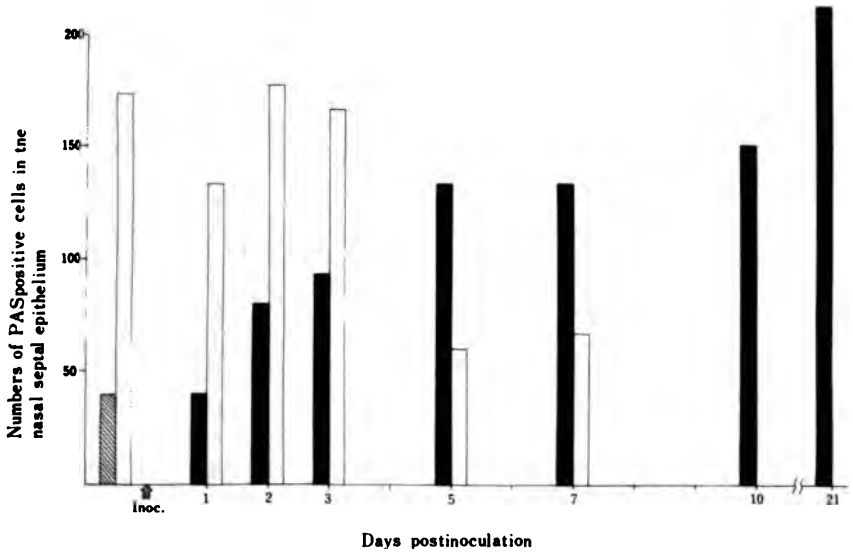


FIG. 5. Diagrammatic summary of differential sequence of pathological changes after virus inoculation wit and without SO_2 exposure. Stippling represents epithelial damage; hatched lines and crosses represent early an later repair; dash lines indicate regenerated mucosae.



5. Changes in the numbers of goblet cells in septal mucous membrane over time. Hatched bar indicates no virus or SO_2; black bar indicates virus alone, no SO_2; white bar indicates SO_2 plus virus.

xposure of tracheal epithelia to low of SO_2 (27) and after intermittent re of bronchial epithelia to heavy trations of SO_2 (14).

histopathology produced in the nose *ıbined SO_2 and virus* in the present nents suggests that progressive alter- f nasal mucosae by SO_2 allowed very proliferation of virus in these tissues. ure, this breakdown in the normal e mechanisms in the nose would leave ver tract tissues vulnerable to effects es, viruses, or combinations of the

rly a systematic experimental study ractions between chronic air pollution spiratory virus infections in nasal tis- and subsequent effects on tracheo- ıial tissues, has not yet been achieved. ally lacking are data on the differen- ects of pollutants on altered produc- f secretory antibody, interferon, and ne.

mary. The mouse-adapted strain of fluenza virus was inoculated intranas- o unanesthetized DS mice which had ontinually exposed to 0.03–0.1 ppm $_2$ for 4 weeks. Histopathological s in nasal tissues showed that inflam- response was more rapid and more severe, and regeneration was initiated sooner, in these mice than in virus-inoculated controls. The HI titer also developed more rapidly and reached higher levels in mice exposed to SO_2 plus virus than in mice receiving virus alone, and antibody to the virus appeared earlier in the exposed mice.

Animals which were exposed to SO_2 and were not given the virus showed a sixfold increase in the number of goblet cells in nasal respiratory epithelial cells, though the olfactory epithelial cells were apparently unaffected.

The results suggest that increased severity of influenza infection after continued exposure to low levels of SO_2 resulted from a progressive alteration of the nasal mucous membranes by SO_2 which then allowed rapid destructive proliferation of subsequently introduced virus. This destruction of nasal tissues would eliminate a major defensive barrier against lower tract disease.

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Received November 5, 1976. P.S.E.B.M. 1977, Vo 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 597-601 (1977)

Angiotensin II Increases Uterine Vascular Resistance in Pregnant and Nonpregnant Rabbits (39726)[1, 2]

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Introduction. That angiotensin II (A-II) produces marked vasoconstriction has been well-documented for most vascular beds including the kidney, intestine, hindlimb, heart, and liver (1-6). In contrast, studies of the effects of A-II on uterine blood flow have generated conflicting findings.

Girard *et al.* (7) using a radiographic technique for detecting changes in distribution of blood flow in pregnant guinea pigs found that intravenous administration of A-II produces virtually no change in uterine blood flow. In contrast, Greiss and van Wilkes (8) reported that A-II significantly increased uterine vascular resistance in pregnant sheep. However, Assali *et al.* (9) and Ladner *et al.* (10) reported that intravenous injections of A-II passively decreased uterine vascular resistance in either pregnant or nonpregnant sheep and dogs. More recently Ferris *et al.* (11), working with pregnant rabbits, and Terragno *et al.* (12, 13), working with pregnant dogs, claimed that A-II decreased uterine vascular resistance and increased uterine blood flow. None of these studies, however, examined the direct effects of A-II on uterine vascular resistance as revealed by injecting A-II into the uterine artery.

To gain insight into this problem we compared the effects of intraarterial injections of A-II on uterine vascular resistance in pregnant and nonpregnant rabbits. Such information is of interest because of the purported role of A-II in local regulation of the uterine vasculature, particularly during pregnancy (11-14).

Methods. Studies were performed with 12 sexually mature New Zealand virgin rabbits (3.2-4.8 kg) and 5 pregnant rabbits (third trimester, 4.0-6.1 kg) anesthetized with sodium pentobarbital (50-100 mg/kg iv), tracheotomized, and artifically ventilated with humidified room air. Supplemental anesthesia was administered through a catheterized jugular vein, and arterial blood pressure (E and M transducer, P-1000) was monitored from the left femoral artery.

The procedure used to study the effects of drugs on uterine vascular resistance was adapted from Ryan *et al.* (15). The uterus and its blood supply was exposed through a midline abdominal incision (Fig. 1). All branches of the right external iliac artery between the iliac bifurcation and the right uterine artery were ligated. The right external iliac artery was ligated immediately distal to the right uterine artery. Ligatures were placed around the right uterine horn where it joined the cervix and around the fallopian tube. The mesometrium was tied in several sections so that blood entered the right uterine horn largely, or solely, through the right uterine artery and drained the horn via the uterine veins which were unaltered. The left uterine horn and its blood supply was kept intact.

Heparin (1500 μ/kg iv) was administered to prevent blood coagulation after all major surgery was completed. Blood from a common carotid artery was diverted through a short (50-60 cm) extracorporeal circuit which contained a precalibrated constant flow peristaltic pump (Sigmamotor, T-8). Blood from the outflow side of the pump

[1] A preliminary report of these findings was presented at the fall meetings of the Federation of American Societies For Experimental Biology, Anaheim, California, April, 1976.

[2] This study was supported by a grant from the Southwestern Ohio Chapter of the American Heart Association.

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ISSN 0037-

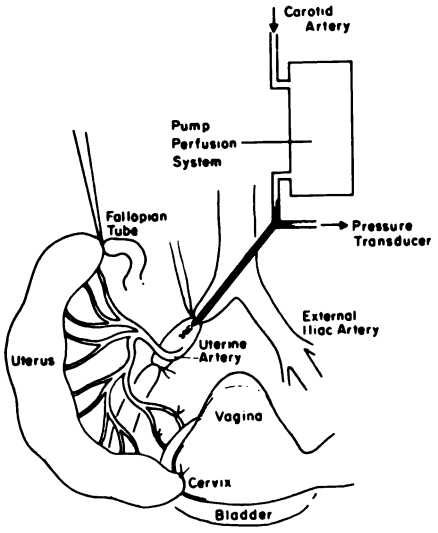

FIG. 1. Schematic representation of the procedure used for isolating and perfusing a segment of the uterine vasculature *in situ*. Technical details are described in Methods.

was used to perfuse the isolated uterine horn via a catheter in the right uterine artery. Uterine perfusion pressure was monitored through a Y-tube placed between the pump and the perfused segment. Flow through the pump, and hence the collateral free uterine horn, was increased gradually until perfusion pressure was within 5% of aortic blood pressure. Preliminary experiments showed that the preparation was stable throughout the experimental period (2–3 hr).

Intra-arterial (ia) injections of A-II (Beckman) were made through the inflow side of the pump to permit thorough mixing with blood (5, 6, 16). The drug was injected rapidly (1–2 sec) in 0.1 ml of saline (pH 7.3–7.4) and 10–15 min elapsed between injections so that tachyphylaxis did not develop (3–6). The primary response to A-II was recorded as the maximal change in perfusion pressure (expressed as a percentage of control perfusion pressure) which occurred prior to any change in arterial blood pressure (5, 6). Control injections of saline (0.1 ml) were performed in each experiment to insure that responses to A-II were not mechanical artifacts.

In three pregnant and four nonpregnant rabbits, responses to A-II were examined in the presence and absence of 1-sar-8-ala-angiotensin II (saralasin, Norwich, Pharmacal), a specific A-II antagonist. The influence of α-adrenergic blockade with phentolamine (Ciba) on responses to A-II and DL-norepinephrine (Winthrop) was evaluated in four nonpregnant rabbits. The local effects of nitroglycerin (Lilly) were also tested to ensure that the vasculature was able to relax. Since uterine blood flow was maintained constant, agonist-induced changes in uterine perfusion pressure were directly proportional to changes in uterine vascular resistance (16).

Uterine responses were expressed as mean values ± 1 SE and the statistical significance of differences between responses in pregnant and nonpregnant rabbits, and between responses in the presence and absence of antagonists were assessed with Student's *t* test. In all experiments the schedule of injections was randomized with respect to the agonist and dose of agonist tested.

India ink was injected into the uterine perfusion system shortly before the termination of each experiment to visualize the distribution of ink through the perfused segment and to determine whether the segment was well-isolated. If a diffuse pattern was observed revealing contamination within the vagina, bladder, or contralateral uterine horn, the experiment was discarded.

Results. Mean arterial blood pressure following all surgical procedures was slightly but significantly ($P < 0.05$), greater in the 12 nonpregnant rabbits (99 ± 6 mm Hg) than in the 5 pregnant rabbits (70 ± 7 mm Hg). Similarly, uterine perfusion pressure was higher in nonpregnant (89 ± 5 mm Hg) than in pregnant (75 ± 4 mm Hg) preparations. However, blood flow through the perfused uterine segments was greater in pregnant (5.0 ± 0.4 ml/min) than in nonpregnant rabbits (3.2 ± 0.3 ml/min; $P < 0.005$). Accordingly, vascular resistance through the perfused segments, calculated from the ratio of uterine perfusion pressure to blood flow through the perfused segment, was markedly and significantly ($P < 0.025$) greater in the nonpregnant population (29 ± 4 mm Hg/ml/min) than in the pregnant population (18 ± 4 mm Hg/ml/min).

'a-arterial injections of A-II consist-produced marked increases in uterine lar resistance (increased uterine perfu-ɔressure) in both pregnant (Table I) onpregnant rabbits (Fig. 2, Table I). nfluence of time elapsed between ter-ion of the response to a given dose of and initiation of a second response to me dose was studied in four nonpreg-rabbits. In response to the first test ion of A-II (1 μg ia) uterine perfusion ire increased to 285 ± 21% of control. ver, responses elicited 2 min (115 ± control) or 4 min (180 ± 13% of ɔl) after completion of the initial re-e were depressed markedly. In con-tachyphylaxis was not evident when terval was lengthened to 8 min (270 ± of control) or 16 min (273 ± 9% ɔl).

ɔoth pregnant and nonpregnant rabbits rictor responses to A-II were rapid in (20–30 sec), attained maximal levels -70 sec after onset, and returned to ɔl levels in 240–300 sec. The responses ly occurred in the absence of changes stemic arterial pressure. However, high doses of A-II (> 0.25 μg) were l, the local responses were often fol-l by a transient pressor response which mall in magnitude (Fig. 2).

ravenous injections of A-II (5 μg), like arterial injections, produced marked ıses in uterine vascular resistance. , intravenous administration of A-II in-ɛd uterine perfusion pressure to 138 ± f control ($P < 0.025$) in nine nonpreg-rabbits, and to 146 ± 12% of control 0.025) in five pregnant rabbits.

ɔckade of A-II receptors with 1-sar-8-

.E I. Effects of Graded Doses of A-II on rine Perfusion Pressure in Pregnant and Nonpregnant Rabbits.

	Uterine perfusion pressure (% of control)[a]	
(μg)	Pregnant	Nonpregnant
02	109 ± 3 (5)[b]	119 ± 4 (5)
06	151 ± 6 (5)	153 ± 9 (9)
25	199 ± 13 (5)	228 ± 17 (9)
00	275 ± 12 (5)	278 ± 16 (6)

alues represent mean response ± SE.
umber of rabbits tested in parentheses.

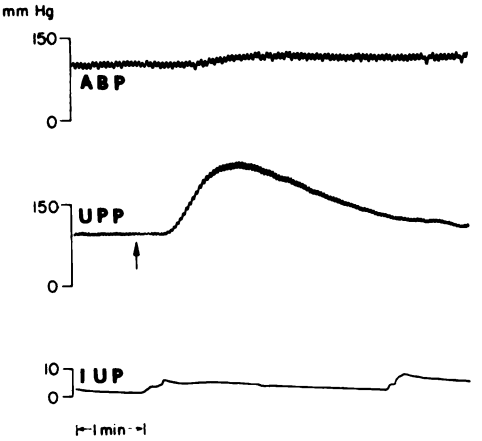

Fig. 2. The effects of A-II (1.0 μg) injected directly into the pump-perfused uterine vasculature of a nonpregnant rabbit at constant flow are shown with respect to arterial blood pressure (ABP), uterine artery perfusion pressure (UPP), and intrauterine pressure (IUP). The time of injection of A-II into the perfusion system is represented by the arrow.

ala-angiotensin II (saralasin, 30 μg/kg ia) virtually abolished constrictor responses to local injections of A-II in either pregnant or nonpregnant rabbits. For example, the mean increase in uterine perfusion pressure produced in response to 0.25 μg of A-II ia was 188 ± 12% of control before saralasin, but only 104 ± 4% of control after saralasin ($P < 0.005$). Similarly, the control uterine vascular response to the same dose of A-II in three nonpregnant rabbits was 195 ± 10% of control, but decreased to 106 ± 3% of control ($P < 0.005$) after administration of saralasin. However, treatment with the A-II antagonist did not influence constrictor responses to norepinephrine in any of the three nonpregnant rabbits examined. Thus, norepinephrine (1 μg ia) increased uterine perfusion pressure to 193 ± 5% of control in the absence of saralasin, and to 205 ± 16% of control after blockade of A-II receptors.

In contrast, blockade of α-adrenergic receptors with phentolamine (1.5 mg/kg iv) in four nonpregnant rabbits significantly attenuated local constrictor responses to norepinephrine but did not alter responses to A-II. For example, norepinephrine (1 μg ia) increased uterine perfusion pressure to 192 ± 11% of control before blockade, whereas

the response was decreased to 127 ± 2% of control ($P < 0.005$) after α-blockade. The responses to A-II (0.06 μg ia) before (168 ± 6% of control) and after phentolamine (165 ± 9% of control) were similar.

Nitroglycerin (600 μg ia), as expected, reduced uterine perfusion pressure in three pregnant (62 ± 4% of control; $P < 0.025$) and three nonpregnant rabbits (72 ± 6% of control, $P < 0.025$).

Discussion. The prominent finding in this study was that A-II always increased uterine vascular resistance when injected either intravenously or directly into the uterine arterial blood supply of either pregnant or nonpregnant rabbits (Fig. 2, Table I).

The present findings contrast with studies reported from other laboratories showing that A-II had no effect on the uterine vasculature of pregnant guinea pigs (7), or that it decreased uterine vascular resistance in pregnant rabbits (11) and dogs (12, 13). Although the reasons for these disparate findings are obscure, contributing factors probably include species differences with respect to guinea pigs, rabbits, and dogs (17, 18) and procedural differences including anesthesia, surgical preparative techniques, and methods used for assessing uterine vascular responses to A-II. For example, Ferris *et al.* (11) used microspheres to assess uterine blood flow and found that prolonged intravenous infusions (30 min) of A-II decreased uterine vascular resistance in near-term pregnant rabbits which had been nephrectomized 24 hr prior to study. This decrease in resistance was reportedly indirect since blockade of β-adrenergic receptors with propranolol abolished the response. In this context, we found that rapid (2–4 sec) intravenous injections of A-II, which reveal the immediate effects of A-II, increased uterine vascular resistance in either pregnant or nonpregnant rabbits. However, because of the procedural differences between these studies a strict comparison of our findings with those reported by Ferris *et al.* (11) is not strictly appropriate.

In the present study, a segment of the uterine vasculature was isolated *in situ* so that it was collateral free and pump perfused with the animal's own arterial blood under conditions of constant flow. In contrast to the procedures used by other investigators, we focused primarily on responses when A-II was injected directly in uterine blood supply. Changes in vascular resistance were directly tional to changes in uterine perfusio sure because flow was constant (5, Local uterine responses occurred and were probably independent of terine reflexogenic mechanisms whic have been elicited when A-II was a tered intravenously (19). Our findi uterine vascular resistance was signi lower in pregnant than in nonpregna bits is consistent with findings repo other laboratories (17, 18) and sugge the preparations were not seriously c mised by the surgical procedures u addition, nitroglycerin significantly r uterine perfusion pressure in pregn nonpregnant preparations showing t vascular beds could relax when stir appropriately. Therefore, the consis servation that A-II increased uterin sion pressure must be due to active v striction of the uterine vasculature.

Uterine vasoconstriction produc A-II was dose dependent and si magnitude for both pregnant and n nant rabbits (Table I). This finding that pregnancy was not associate change in the responsiveness of the vasculature to A-II. However, this sion should be viewed with caution uterine vascular resistance was pregnant animals and because prec mation on the E_{50} for uterine vas sponses to A-II in either pregnan pregnant rabbits is lacking.

The vasoconstrictor responses were probably ascribable largely interactions between the agonist cific receptors for A-II in uterine resistance vessels and were indep interactions between A-II and α-a mechanisms in the uterine vascula conclusion is supported by two tions. First, responses to A-II were abolished during blockade of A-II with 1-sar-8-ala-angiotensin-II wh sponses to norepinephrine were u Second, responses to A-II were during α-adrenergic blockade with amine, whereas responses to no rine were significantly attenuated.

iis study does not permit an
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flow since the drugs were ad-
levels higher than those en-
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Jterine vascular responses to
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Received July 16, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 602-605 (1977)

Isoproterenol-Induced Diuresis in Turkeys[1] (39727)

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We reported earlier that sc administration of *d,l*-isoproterenol sulfate dihydrate and *d,l*-isoproterenol hydrochloride (isoproterenol; Aldrich Chemical Co., Milwaukee, Wis.) to turkeys stimulated the flow of urine (1). The observation was surprising because isoproterenol, administered to mammals, has a strong antidiuretic effect (2-7). The mode of action in mammals may be directly on renal tubules to stimulate cAMP and thus mimic the effect of antidiuretic hormone (ADH) (6, 8, 9), or by way of stimulating ADH secretion (10, 11).

Because of the striking difference in the renal responses of mammals and the turkey to isoproterenol, the effects of this β-adrenergic agonist on renal hemodynamics, fluid and electrolyte balance, and systemic circulation were studied. The results indicate that the isoproterenol-induced diuresis is associated with an increased glomerular filtration rate (GFR) and increases in free-water clearance (C_{H_2O}).

Methods. Six, 5-month-old, broad-breasted white turkey hens with a mean weight of 7.6 ± 0.2 kg were studied. They were fed a commercial poultry ration and watered *ad libitum* in a communal pen for 6 weeks before the experiment began. Each bird was placed in a stainless steel cage for 16 hr without food or water before measuring renal clearances.

Experimental protocol. At the outset of experiments on individual birds, 480 mg of *p*-amino hippuric acid (PAH) and 2.7 g of creatinine (Cr) in 30 ml of distilled H_2O were injected sc into the neck. The bird was then restrained on a dog board and the head was covered with a towel. Birds usually lay quietly during the 2.5 hr procedure without use of anesthesia. In the 30 min before clearance measurements were started, the cloaca was emptied by a combination of flushing with saline, wiping with saline-moistened gauze squares, and aspiration (see below). Cloacal emptiness was determined visually with the use of a canine vaginal speculum. After gently stretching the cloacal orifice with the finger, urine emission from the ureters was seen and was collected at 4- or 5-min intervals in a filtering flask trap by aspiration with a vacuum pump. The aspiration tube (NCG oxygen catheter, National Cylinder Gas, Chicago, Ill.), attached to rubber tubing, contained perforations which excluded bits of feces. Between the sequential clearance measurements, the system was rinsed with 10 ml of isotonic saline. Later, urine volumes and Na excretion were corrected for this amount.

Thirty minutes after the Cr-PAH injection, the following clearance measurements were made: creatinine (C_{Cr}), PAH (C_{PAH}), sodium (C_{Na}), osmolal (C_{Osm}), and free water (C_{H_2O}). Ten milliliters of venous blood were collected by venipuncture from the wing vein midway in each 30-min interval. The concentration of the respective solutes in this sample was used to calculate clearances.

Creatinine and PAH were assayed by routine methods (12), Na and K were assayed by flame photometry, and osmolality was assayed by freezing point depression. Data were statistically analyzed by the method of least significant difference (13).

Treatments. Experiment 1. After an initial control clearance determination, each bird was injected sc with *d,l*-isoproterenol hydrochloride or isotonic saline in a 1-ml volume. After the injection, clearances were determined sequentially three times at 30-

[1] Florida Agricultural Experiment Station Journal Series 119. Supported in part by Grants HL-14526-05 and HL-17909 from the National Heart and Lung Institute.

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ISSN 0037-9727

ıtervals. The injectate was varied so alf the birds received saline first and :ceived isoproterenol first. On another he treatment was reversed and clear- measurements were repeated. Hence, ffects of saline and of isoproterenol measured in each bird.

ieriment 2. Four of the birds were :d further (technical problems pre- d study of the other two). Two birds used as saline-injected controls and vere injected with isoproterenol. The protocol was used as in experiment 1 t that the carotid artery was cannu- with Clay Adams PE 160 polyethylene ; under local anesthesia, and systolic, ılic, mean, and pulse pressures were led using a Narco Bio-Systems poly- and a P-1000 pressure transducer. 30-min control periods preceded ad- tration of *d,l*-isoproterenol hydro- de (400 μg/kg calculated as salt). Af- rds, measurements were made and av- d in each of the three 30-min intervals.

ults. Experiment 1. Isoproterenol, at a of 400 μg/kg body wt stimulated urine ction (Table I). This effect occurred tently and began within 10 min of the ion, reached a maximum within 1 hr, ıen gradually declined though remaining above control levels until the experiment ended. In preliminary experiments on other birds 180 μg of *d,l*-isoproterenol hydrochloride/kg body wt stimulated urine production in only one of three trials, and 90 μg/kg stimulated urine flow moderately in one trial. Since 400 μg of isoproterenol/kg body wt consistently induced diuresis, the present experiments were performed using this dosage.

The C_{Cr} increased significantly above the level of saline-treated controls after administration of 400 μg of *d,l*-isoproterenol/kg body wt/(Table I). The C_{PAH} in both control and isoproterenol-treated birds decreased during the treatment period.

Both C_{Na} and C_{Osm} increased significantly above the levels of saline-treated controls (Table I). The C_{H_2O} was negative for saline-treated turkeys throughout, but became positive in the first clearance period following administration of isoproterenol and then was highly variable (Table I).

Mean carotid arterial pressure declined in both groups even during the control period, but declined more drastically after administration of isoproterenol (Table II). Mean carotid pressure then stabilized at approximately 60 mm Hg while that of saline-injected birds declined progressively. The de-

TABLE I. RENAL FUNCTION IN TURKEYS INJECTED SC WITH 400 μg OF *d,l*-ISOPROTERENOL HYDROCHLORIDE/kg BODY WEIGHT.[a]

Injectate	Preinjection Control	Postinjection (min) 30	60	90
lume (μl/kg/min)				
	31 ± 4	27 ± 2	25 ± 2	25 ± 2
ıterenol	36 ± 4	218 ± 18*,**	352 ± 38*,**	263 ± 38*,**
ıe clearance (ml/kg/min)				
	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
ıterenol	1.8 ± 0.1	2.5 ± 0.1*,**	2.5 ± 0.1*,**	2.4 ± 0.1*,**
hippuric acid clearance (ml/kg/				
	28 ± 3	24 ± 3	22 ± 2*	19 ± 2*
ıterenol	32 ± 3	24 ± 1*	22 ± 2*	17 ± 1*
clearance (μl/kg/min)				
	18 ± 5 (5)	19 ± 3	24 ± 2	27 ± 6
ıterenol	30 ± 3*,**	174 ± 18*,**	318 ± 27*,**	228 ± 36*
clearance (μl/kg/min)				
	61 ± 7 (4)	50 ± 5 (5)	57 ± 5 (4)	44 ± 7 (4)
ıterenol	60 ± 4 (4)	196 ± 16 ± (5)*,**	394 ± 41*,**	248 ± 29*,**
er clearance (μl/kg/min)				
	−31 ± 4 (4)	−24 ± 6 (5)	−33 ± 4 (4)	−20 ± 6 (4)
ıterenol	−24 ± 3 (4)	+34 ± 8 (5)*,**	−40 ± 28	+14 ± 20

an ± SE. $N = 6$ unless indicated differently in parenthesis. These differences result from insufficient sample volumes.
nificantly different from preinjection control ($P < 0.01$).
gnificantly different from saline injected birds ($P < 0.01$).

TABLE II. SYSTEMIC CIRCULATORY CHANGES IN TURKEYS INJECTED SC WITH 400 μg OF *d,l*-ISOPROTERENOL HYDROCHLORIDE/kg BODY WEIGHT.[a]

	Preinjection control (min)		Postinjection (min)		
	30	60	30	60	90
Mean carotid pressure (mm Hg)					
Saline	180	170	161	148	142
Isoproterenol	188	134	60	58	58
Carotid pulse pressure (mm Hg)					
Saline	59	55	45	34	27
Isoproterenol	71	53	67	60	56
Carotid pulse rate (beats/min)					
Saline	225	209	209	204	200
Isoproterenol	183	183	228	230	222

[a] Mean of two saline-injected and two isoproterenol-injected birds. Each value represents mean of measurements made at 5-min intervals over 30 min.

cline in blood pressure of saline-treated birds may have been related to prolonged restraint and/or volume depletion due to blood sampling.

In controls, pulse pressures declined more or less progressively. This did not occur after isoproterenol because reductions in diastolic pressure were proportionately larger than those of systolic pressure. The pulse rate increased moderately after isoproterenol.

Discussion. In turkeys, isoproterenol induced a diuresis in contrast to the antidiuresis reported for certain mammals (1–7). This effect in turkeys might result from direct effects on the kidneys, e.g., altered renal hemodynamics, nephron recruitment, or changes in tubular reabsorptive mechanisms, or it might result from indirect renal effects, e.g., decreased ADH secretion or alterations in the systemic circulation.

Apparently the effects of isoproterenol on the systemic circulation are similar to those described for mammals, i.e., increased myocardial contractility and decreased peripheral vascular resistance (14). Reduced vascular resistance was indicated by the reduction in mean arterial pressure, and by a proportionately larger decrease in diastolic than in systolic pressure. Increased pulse pressure may indicate a greater myocardial contractility leading to an increase in stroke volume. Together with the increased pulse rate, this is indicative of increased cardiac output (15).

The C_{Na} and C_{Osm} consistently increase after isoproterenol. This could result from either increased filtered loads or decreased tubular reabsorption of Na and other osmotically active solutes. Since C_{Cr} increased and Na concentration in plasma usually increased, it is likely that increases in filtered load account for the increases in C_{Na} and C_{Osm} and suggest the diuresis was at least partly osmotic in origin. Such increases in filtered loads could result either from increased filtration rates of individual nephrons, or from recruitment of additional nephrons.

After isoproterenol, C_{H_2O} increased initially and then became highly variable. Concomitantly the urine osmolality fell, the greatest reduction being in the first clearance period. Birds possess two nephron populations, a nonconcentrating reptilian type and a concentrating mammalian type (16). Hence, the C_{H_2O} changes are interpretable in terms of increased reptilian nephron activity, due either to recruitment of more nephrons or to increased filtration by individual nephrons. The C_{H_2O}, however, often is used as an indirect measure of ADH activity. Conceivably, the initial increase in C_{H_2O} resulted from ADH inhibition. Such an effect would, however, be in striking contrast with the effect of isoproterenol in mammals, i.e., increased ADH activity (6–11). Moreover, the isoproterenol-induced hypotension, the diuresis-induced volume depletion and increased plasma osmolality are likely

stimulated ADH secretion.
tinine is cleared by glomerular filtra-
d by renal tubular secretion in birds
lowever, the measured increase in
likely to reflect primarily increased
ular filtration. Plasma creatinine con-
ion, though elevated from normal,
ntly decreased with time. Under
nditions, creatinine cleared by tubu-
etion would be expected to remain
ged or decline due to the saturation
of the creatinine secretory mecha-
7). The increments in C_{Cr}, therefore,
ly reflect increases in GFR. Histo-
vidence, viz, arteriolar and tubular
on, supports such an interpretation

changes in C_{Cr} could result from
l resistance (dilatation) in either the
t arteriole or glomerular capillaries,
ed resistance (constriction) in the ef-
arteriole, or dilatation of Bowman's
or the tubule, thus increasing the
ular-capsule hydrostatic pressure
t which drives filtration. This latter
annot be ruled out, but there is little
nce for such an effect. More likely,
re preglomerular or glomerular β-
rs which are responsible for de-
l vascular resistance and increased
ular flow due to recruitment of more
uli and/or increased filtration by indi-
glomeruli. Constriction of the vessels
an affect usually associated with β-
rgic stimulation, and it is therefore
y that there was increased efferent
ar resistance.

nary. The isoproterenol-induced di-
n turkeys is associated with an in-
in C_{Cr} and C_{H_2O}. The effect is proba-
diated, in part, by β-adrenergic re-
in the afferent arteriole which lead
o recruitment of reptilian-type neph-
rons or more filtration by individual neph-
rons. In other respects the effects of isopro-
terenol in turkeys resembles those known to
occur in mammals.

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Received September 13, 1976. P.S.E.B.M. 1977, Vol. 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 606-609 (1977)

High Gain Placental Cardiovascular Control[1] (39728)

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The ventilation-perfusion ratios in the mammalian lung are partially stabilized by local feedback loops so that changes in alveolar ventilation cause changes in the adjacent pulmonary circulation (1) and changes in the pulmonary circulation affect the ventilation of the adjacent alveoli (2). These mechanisms assist in the stabilization of ventilation-perfusion ratios and aid in the transfer of respiratory gases. Like the lung the placenta is an organ of exchange. The uterine blood flow is the analog of the alveolar ventilation and the umbilical blood flow is the analog of the pulmonary circulation. The arguments which apply to the effect of ventilation-perfusion ratios on the pulmonary exchange of respiratory gases also apply to perfusion-perfusion ratios and placental exchange (3). For this reason we attempted to demonstrate the presence of mechanisms linking the uterine and umbilical blood flows. We used a preparation in which the flow in the perfused umbilical circuit was changed while the responses of the adjacent uterine flow were measured.

Methods. Eight near-term goats were used in this study. The gestational ages ranged from 120 to 130 days. The mother was anesthetized with intravenous pentobarbital. A tracheotomy was performed and catheters were placed in a maternal femoral artery and uterine vein. A small incision was made in a relatively cotyledon-free area of the uterus. The umbilical cord was located and the cord vessels were catheterized so that it was possible to pump blood into the umbilical arteries and to recover the venous drainage. In this way the placenta could be perfused for several hours. The mother's body was lowered into a bath containing 150 liters of Krebs saline solution with the bicarbonate replaced by chloride, so that the weight of the uterus and contents did not rest upon the inferior vena cava. The placental perfusion circuit consisted of a primary perfusion pump and an additional pump which lifted the venous drainage into bubble oxygenators which equilibrated the blood with a gas mixture containing CO_2, O_2, N_2, and acetylene (C_2H_2). The mother breathed a gas mixture containing O_2, N_2 and nitrous oxide (N_2O).

The umbilical blood flow ($\dot{Q}_f$) was determined by the setting of the primary perfusion pump, which was calibrated against timed collections of venous outflow. Blood samples were drawn from the maternal artery (MA), uterine vein (MV), umbilical perfusate (FA), and from the umbilical veins (FV). The N_2O and C_2H_2 concentrations (C) in the blood samples were determined by gas chromatography (Beckman GC-2A with blood gas accessory). The uterine blood flow ($\dot{Q}_m$) was calculated according to the equation

$$\dot{Q}_m = \dot{Q}_f(C_{FA} - C_{FV})/(C_{MV} - C_{MA})$$

for both the C_2H_2 data and the N_2O data. Data were considered to be acceptable when the calculated results for $\dot{Q}_m$ from the N_2O and C_2H_2 values differed by no more than 10%. The uterine blood flow was recorded as the mean of the paired N_2O and C_2H_2 data.

The placenta was perfused for 30 min at a fixed rate. Blood samples were then taken from the four sites (MA, MV, FA, FV). The umbilical blood flow was then changed and maintained at its new level for a further 30 min, at which time a second set of blood samples was taken. In some preparations a third umbilical blood flow was studied and a third set of data were obtained.

Full details of the surgery, assay, the per

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ISSN 0037-97

fusion circuit, and the maintenance of physiological gas concentrations in the perfusion blood are provided elsewhere (4).

The respiratory gas status of the blood entering the umbilical arteries was: PO_2, 16 mm Hg; PCO_2, 49 mm Hg; and pH, 7.06. The equivalent values for maternal arterial blood were PO_2, 120 mm Hg; PCO_2, 31 mm Hg; and pH, 7.37. These values are similar to established values for the goat (4). The blood in the perfusion circuit was drawn into heparinized containers 12 h before the perfusion. The donors were unanesthetized nonpregnant goats. The average hemoglobin concentration in the blood of the pregnant goats was 10.3 g% and that of the blood from the donor goats was 9.4 g%. The uterine venous pressures were not measured. The umbilical venous pressures were adjusted to a value of approximately 5 mm Hg by raising or lowering the venous reservoir.

All data reported were obtained from preparations in which the maternal and fetal blood values of pH, PO_2 and PCO_2 were within normal limits.

Results. Data from eight animals are presented in Table I. Fourteen changes in umbilical perfusion rate could be correlated with changes in calculated uterine blood flow. In 13 of the 14 observations a change in umbilical perfusion rate induced a change in the same direction in the uterine blood flow. The ratio of the second umbilical flow to the original is also given in Table I, together with the ratio of the corresponding uterine blood flows. The changes in uterine blood flow were quite large in most cases. The χ^2 test was applied against the assumption that, of the 14 changes in uterine blood flow, 7 would change in the same direction as the umbilical flow and 7 would change in the opposite direction. The resultant χ^2 value of 8.64 indicated that changes in the umbilical blood flow caused the uterine blood flow to change in the same direction ($P < 0.005$).

The values labeled 2c and 2d in Table I were obtained simultaneously because goat 2 carried twins and both placentas were perfused. In 2d the umbilical perfusion rate was increased and the uterine flow to that placenta also increased. In 2c, the umbilical perfusion rate was decreased and the uterine flow to that placenta decreased. Hence the maternal flow to these twin placentas followed the umbilical flows even though this required the placentas to react simultaneously in opposite directions.

Discussion. In these experiments the uterine blood flow changed in the same direction as the change in umbilical perfusion

TABLE I. EFFECT OF CHANGING FETAL PLACENTAL BLOOD FLOW (F) ON THE MATERNAL PLACENTAL BLOOD FLOW (M).[a]

Goat	Fetal weight (kg)	Blood pressures (mm Hg)			Blood flows (ml/min)				Flow ratios		Direction[b]
		F1	F2	Maternal	F1	F2	M1	M2	F1/F2	M1/M2	
1a	1.93	69	59	65	300	200	397	310	0.67	0.78	Same
1b	1.93	60	90	65	200	350	310	525	1.75	1.69	Same
2a	1.51	94	45	100	154	54	1540	242	0.35	0.16	Same
2b	1.51	45	74	97	54	140	242	507	2.59	2.10	Same
2c	1.51	74	49	88	140	65	507	285	0.46	0.56	Same
2d	1.00	59	85	89	43	118	280	885	2.74	3.16	Same
3a	2.36	89	77	100	190	110	560	525	0.58	0.94	Same
4a	–	95	108	110	138	168	860	1050	1.22	1.22	Same
5a	2.22	78	54	–	290	210	604	122	0.72	0.20	Same
6a	1.49	39	58	81	215	257	423	514	1.20	1.22	Same
6b	1.49	58	75	79	257	303	293	354	1.18	1.21	Same
7a	1.50	50	48	85	100	97	523	508	0.97	0.97	Same
8a	1.84	49	55	84	100	116	602	628	1.16	1.04	Same
8b	1.84	50	68	83	116	213	628	492	1.84	0.78	Opposite

[a] The fetal flow is changed from F1 to F2 and the maternal flow responded by changing from M1 to M2. The fetal weights, perfusion pressures, and maternal arterial pressures are also given.

[b] Same means that the uterine blood flow changed in the same direction as did the fetal blood flow. Opposite means that it did not.

rate. This observation supports the hypothesis that local reflex mechanisms within the placenta stabilize local perfusion-perfusion ratios, and that these mechanisms are analogous to those described as stabilizing ventilation-perfusion ratios in the lung.

Differing results have been reported by other investigators. Raye *et al.* (5) observed no rapid change in uterine blood flow in sheep when the fetus was killed, a result which may be explained by the work of Grant *et al.* (6) who showed that the ability of the local mechanisms to stabilize the ventilation-perfusion ratio in the lung is greatest in the physiological range but is relatively poor outside this range. If such a mechanism exists in the placenta it would not be apparent after fetal death. It is also possible that the mechanism which matches uterine blood flow to umbilical blood flow requires a continued umbilical circulation.

Berman *et al.* (7) reported that they observed no changes in the uterine blood flow in response to changes in the umbilical blood flow. In their experiments the umbilical blood flow was changed by partial occlusion of the umbilical cord and the uterine flow was monitored for 60 sec. It is improbable that responses of the type which are suggested here could be detected in so short a time span. Tucker and Reeves (8) have shown that the resistance of the pulmonary circulation is maximal 4 min after instituting alveolar hypoxia.

Grant *et al.* (6) report the open loop gain for their system. In our preparation such sophisticated calculations are hardly appropriate. The changes we induced were large and the number of observations we could make in one animal were extremely limited. When such calculations are applied to the data given in Table I it becomes apparent that the open loop gains are extremely high. In fact, in many cases the uterine flow appears to overshoot, the compensation being greater than that needed to return the perfusion-perfusion ratio to its control state. The presence of high gain control systems in the placental circulation is an unexpected finding and may reflect the release of the placental vascular bed from fetal control in these perfused preparations. The fetus possesses several mechanisms such as catecholamine release and the renin-angiotensin system (9, 10), whereby cardiovascular control can be achieved. These endogenous control systems may damp out many disturbances initiated from the placental vascular bed but would not be observed in the perfused placenta.

We must, however, add a warning note. These observations were made under very artifical circumstances involving a perfused placenta which means that the blood flows and pressures were different from those observed under physiologic circumstances. Nevertheless, they suggest the existence of a mechanism which, under some conditions, may operate to balance the umbilical and uterine blood flows.

Summary. The placentas of eight near-term goats were perfused with blood containing C_2H_2. The adult goats breathed gas containing N_2O. Catheters were placed in a maternal femoral artery and uterine vein. Knowledge of the perfusion rate and N_2O and C_2H_2 concentrations in the maternal artery, uterine vein, umbilical arteries, and umbilical veins, permitted the calculation of the uterine blood flow.

The uterine flow was calculated before and after a change in the rate of the umbilical perfusion. Fourteen such observations were made. In 13 cases the uterine flow changed in the same direction as did the umbilical flow ($P < 0.005$). The magnitude of the change in uterine flow indicated that the mechanism whereby the change was mediated had a gain of greater than 1.

This work was supported by Grants PHS HE05499, HE06336, and HD2313 and by the Oregon Heart Association.

The authors wish to acknowledge the assistance of Mr. J. E. Welch and Mr. R. Ackley in the conduction of the experimental protocols.

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Received September 2, 1976. P.S.E.B.M. 1977, 154.

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 610-614 (1977)

The Influence of Age and Fasting on Serum Hormones in the Lean and Obese Zucker Rat[1] (39729)

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During the onset of spontaneous obesity, a series of hormonal changes occur which may or may not be essential to development of excessive adipose tissue mass. Previous workers have reported that hormone levels are altered in the obese Zucker rat when compared to the lean littermate (1-4). We have found certain metabolic changes in the obese Zucker rat which could have been induced by an endocrine imbalance (5, 6). For example, an increase in amino acid catabolism (7) and a decrease in nitrogen retention (6) may be caused by a decrease in growth hormone levels, an increase in corticosterone levels, or a combination of these two.

To initially investigate the role of the endocrine system in the development of genetically induced obesity, we wanted to characterize the changes which occur in serum levels of hormones during the dynamic phase of weight gain in the obese Zucker rat. Observations on serum levels of immunoreactive growth hormone, prolactin, thyroid-stimulating hormone, and corticosterone of lean and obese rats between 5 and 11 weeks of age are reported here.

Materials and methods. Experimental animals used in this study were from our colony of Zucker obese rats (8), which was maintained by mating heterozygotes (Fa/fa). All animals were housed in an air-conditioned room (25°) under control conditions of light (12 hr on, 12 hr off) and were fed laboratory chow (Purina) and water *ad libitum*. To study the effects of fasting on serum hormone levels, lean and obese male littermates were either fasted for 24 hr or fed. All rats were sacrificed by decapitation at the same time of day (between 9:00 and 10:00 a.m.) to minimize the effects of diurnal fluctuation of hormone levels. To study the effect of age on serum hormone changes in lean and obese rats, the rats were allowed feed and water *ad libitum* and sacrificed as described above at ages 5, 7, 9, and 11 weeks. Blood was collected from the trunk into centrifuge tubes and allowed to clot. Serum was separated by centrifugation (2000*g* for 20 min) and kept at −20° until assayed.

Insulin, TSH, prolactin, and growth hormone levels were determined using the double antibody technique of Hales and Randle (9) and modification (10) of the iodination procedure of Greenwood *et al.* (11). Materials utilized in the TSH, prolactin, and growth hormone assays were kindly supplied by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases, National Institute of Health. Standards for the TSH, prolactin, and growth hormone assays were NIAMD-Rat TSH-RP-1, NIAMD-Rat Prolactin-RP-1, and NIAMD-Rat GH-RP-1, respectively. Porcine insulin obtained from the Lilly Research Laboratories, Indianapolis, Ind. (615-D-63-10) was used as the standard for the insulin assay.

Growth hormone and prolactin used for radioiodination were dissolved in 0.01 *M* NH_4HCO_3 buffer (12) at pH 8.3. Growth hormone and prolactin standards were dissolved in 0.01 *M* NH_4HCO_3 at pH 8.3 to a dilution of 1 μg/ml and frozen. Just before use, the standards were further diluted to the desired concentration with phosphate-buffered saline containing 1% egg white (PBS-1%EW). Insulin and TSH used for radioiodination were dissolved in distilled water. Insulin and TSH standards were dissolved in PBS-1% EW.

Anti-porcine insulin (64-104-16) was ob-

[1] Authorized for publication on July 6, 1976 as paper 5116 in the journal series of the Pennsylvania Agriculture Expeirment Station. Supported in part by NIH Grant HD 07090-04.

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ISSN 0037-9727

tained from Miles Laboratory, Kankakee, Ill. Anti-monkey γ-globulin used in the growth hormone assay was obtained from Antibodies Inc. Davis, Calif. Anti-guinea pig γ-globulin used in the insulin assay and anti-rabbit γ-globulin used in the TSH and prolactin assays were produced in sheep at The Pennsylvania State University.

Corticosterone was determined by competitive binding radioassay (13) using female nonpregnant rhesus monkey plasma as a source of binding globulin. Analysis of variance and unpaired t test were computed according to Steel and Torrie (14).

Results. Table I shows the effects of fasting on serum hormone levels in lean and obese rats. Insulin levels are depressed in both lean and obese rats by fasting, but still remain elevated in the obese when compared to the lean rat. Growth hormone levels were reduced by fasting in the obese rat but not in the lean rat. Prolactin and TSH were not affected by fasting in either group of animals. Corticosterone was elevated by fasting in both lean and obese rats.

Growth hormone levels increased dramatically with age in lean rats, whereas the obese rat showed only a marginal increase at 9 weeks of age (Fig. 1). Strosser and Mialhe (15) recently reported similar increases in serum GH in normal rats between 20 and 80 days of age. They concluded from their studies that metabolic clearance rate of GH was independent of serum concentration and that serum concentration depends primarily on its secretion rate from the pituitary.

Prolactin levels were higher ($P < 0.01$) in lean rats at 5 and 7 weeks of age. By 11 weeks lean and obese rats had similar levels of prolactin (Fig. 2). The prolactin levels reported here are similar to those reported in decapitated rat blood between the ages of 20 and 70 days in Holtzman male rats.

Serum levels of TSH increased with age in both lean and obese rats. At 9 weeks of age

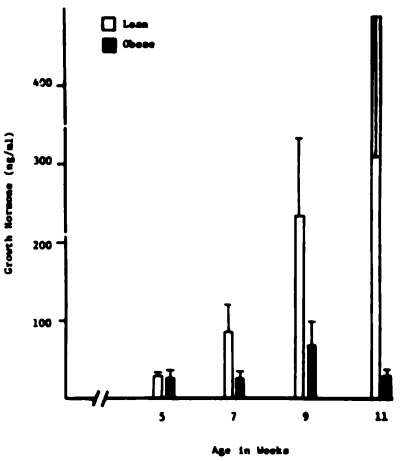

FIG. 1. Serum levels of immunoreactive growth hormone in lean and obese rats at various ages.

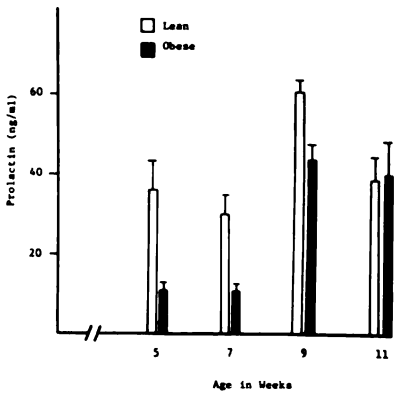

FIG. 2. Serum levels of immunoreactive prolactin in lean and obese rats at various ages.

TABLE I. THE EFFECT OF FASTING ON SERUM HORMONE LEVELS IN LEAN AND OBESE ZUCKER RATS

Hormone	Lean		Obese	
	Fed	Fasted	Fed	Fasted
Insulin (μU/ml)	120 ± 36[a]	18 ± 2*	492 ± 78	175 ± 18*
Growth hormone (ng/ml)	395 ± 149	477 ± 125	189 ± 66	30 ± 4*
Prolactin (ng/ml)	13.4 ± 2.5	15.0 ± 2.0	18.2 ± 6.4	13.2 ± 1.6
Thyroid-stimulating hormone (ng/ml)	31.87 ± 4.99	26.60 ± 1.32	28.7 ± 2.98	32.10 ± 3.49
Corticosterone (μg/100 ml)	8.80 ± 4.25	28.45 ± 10.99*	12.16 ± 4.57	38.66 ± 2.95*

[a] Mean ± SE for five animals.

* Means from fasted animal are significantly different from fed ($P < 0.05$).

serum levels of TSH were higher ($P < 0.01$) in lean rats than in obese rats (Fig. 3).

Corticosterone levels were highest at 5 weeks of age in both lean and obese rats and decreased with age (Fig. 4). Obese rats had higher levels of corticosterone at 11 weeks of age.

Discussion. Some endocrine alterations may be secondarily induced by adaptations to the obese state (17–20). This report describes hormone levels in lean and obese rats during the period of rapid weight gain and obesity onset. It is apparent that some endocrine changes observed in obese Zucker rats are caused by the obese state and are probably not directly involved in the metabolic lesions associated with this type of obesity (1, 3, 7, 21).

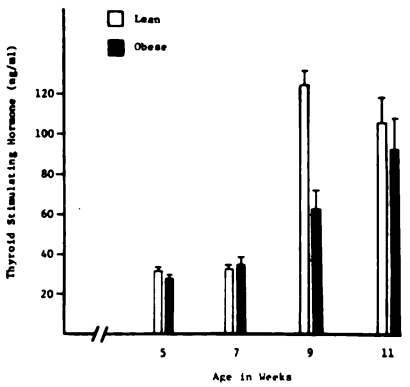

FIG. 3. Serum levels of immunoreactive thyroid-stimulating hormone in lean and obese rats at various ages.

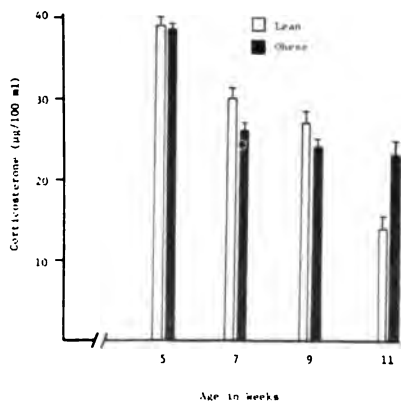

FIG. 4. Serum levels of corticosterone in lean and obese rats at various ages.

Plasma IRI levels were reduced by fasting in both lean and obese rats, and may be elevated in the genetically obese rat because of the hyperphagia associated with this syndrome. In support of this idea, Thenen and Mayer (22) showed that VMH-lesioned hyperphagic rats and the Zucker obese rat have comparable serum IRI levels. However, two types of evidence argue against this theory. We have found that pair-feeding the obese to lean rats did not eliminate hyperinsulinemia (23), and Zucker and Antoniades (1) showed that hyperinsulinemia is present after excessive accumulation of fat and that serum IRI of "fatties" fell to normal levels only when the rats have been fasted long enough to bring their body lipid down to normal.

Pituitary size is reduced in the obese rat (24) and may be the primary lesioned site causing decreased muscle growth (25), reproductive failure (26), and defective regulation of food intake in response to cold stress (27). The lower levels of growth hormone found in this study may explain the impaired muscle development (25) and decreased nitrogen retention (6) found during the dynamic phase of weight gain. Depressed serum growth hormone levels have been reported in obese hyperglycemic mice (28, 29) and hypothalamically induced obesity in rats (30) and mice (31). Elevated levels of lipids and blood glucose, often a part of the obese syndrome, may be involved in depression of serum growth hormone levels (32, 33). Growth hormone response to provocative stimulation is blunted in obese subjects but returns to normal after weight reduction (17, 18). Blackard *et al.* (32) have shown that intravenous injection of soybean oil emulsion inhibited insulin-induced plasma growth hormone elevation in monkeys. From these observations, it would appear that metabolic aberrations which result in excessive lipid synthesis and plasma lipids may produce a secondary lesion in the pituitary resulting in decreased output of growth hormone. The lower levels of growth hormone may in turn result in impaired somatomedin output (34, 35) and muscle development (25, 36) found in various types of obesities.

A role as a lipogenic factor has been im-

ed for prolactin in the following stud-
ats implanted with prolactin secretory
s increase body weight and fat deposi-
37); in the migratory white throated
ow, prolactin was shown to act in a
oral relationship with cortisol to stimu-
premigratory fattening (38); and re-
we showed that, by inhibiting prolac-
ecretion, the lipogenic rate was de-
ed in the quail (39). In the present
prolactin levels were depressed during
arly stages of obesity but were normal
g the later stages. Similar results have
reported for the obese hyperglycemic
e (29). While these data suggest a dif-
ce in prolactin level between lean and
animals, more information on the
al pattern of prolactin and glucocorti-
levels is required for implication of
hormones as causal agents in the ex-
e accumulation of fats.
patic amino acid catabolism and in-
ed urinary nitrogen (6, 7) suggest that
corticoids may be involved in the de-
ment of obesity in the Zucker rat.
presented in this paper suggest that an
tion in glucocorticoids occurs during
ter stages of obesity and is probably a
dary adaptation to the obese condi-

rk and co-workers (24) suggested from
studies that the Zucker "fatty" had
ular control of TSH secretion. In this
t, plasma TSH levels were depressed
e obese at 9 weeks of age only. A
ession in TSH secretion and thyroid
ion could lead to a decrease in basal
bolic rate and thereby increase the en-
available for fatty acid synthesis. How-
we observed no significant difference
ergy required for maintenance of lean
obese rats (6).
is study shows changes in serum hor-
levels during a period of very rapid
th. In comparison to the lean litter-
, the obese rat gains weight primarily in
orm of fat during this period. This ge-
ally induced shift in dietary energy uti-
on may be mediated through an altera-
in endocrine function. The data pre-
d suggest a number of potential mecha-
by which muscle growth is impaired
adipose cell development is stimulated
in the obese Zucker rat.

Summary. Genetically obese Zucker rats (fa/fa) and their nonobese littermates (Fa/?) were studied during the active phase of obesity onset to characterize serum levels of immunoreactive growth hormone, prolactin, thyroid-stimulating hormone, and corticosterone. The effect of fasting on serum hormone levels in lean and obese rats was also investigated. Fasting reduced insulin levels in both lean and obese rats but the fasted levels in the obese rat were still 9 to 10 times higher than the lean littermates. Growth hormone levels were lower in the obese and were reduced by fasting. In the lean rat serum growth-hormone levels increased with age (25 to 480 ng/ml); however, the obese rat showed only marginal increases (25 to 65 ng/ml) during the same period. Corticosterone levels decreased with age and were higher in the obese rat at 11 weeks of age only. Thyroid-stimulating hormone increased with age and was lower in the obese rat at 9 weeks of age. During 5 and 7 weeks of age, serum prolactin was decreased in the obese rat but was similar by 11 weeks of age. These changes are discussed in relationship to their potential role in excessive lipid deposition in genetically induced obesity.

The radioimmunoassay kits for rat growth hormone, prolactin, and TSH were supplied through the generosity of the Hormone Distribution Office, NIAMD, NIH, Bethesda, Maryland.

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Received July 23, 1976. P.S.E.B.M. 1977, Vol. 154.

I OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 615-620 (1977)

ıunomodulator-Induced Resistance Against Herpes Simplex Virus[1] (39730)

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ıduction. Viral infections can be in- by treatment of animals with a vari- mmunomodulators (1-6). The mech- of antiviral action of immunomodu- however, have been defined in only a ıtances. The protective effects of po- nosinic-cytidylic acid and of tilorone hloride appear to be due to the sys- ıroduction of high levels of interferon ıvestigations have not been able to ıh a similar role for interferon in the al effects of synthetic polyanionic im- ıodulators such as pyran and chlorite- :d carboxyamylose (COAM), but uggested that cells of the activated ɔendothelial system might be in- (1, 4). Pyran, as well as biologic omodulators such as *Mycobacterium* ıtrain BCG and the killed vaccine of *:bacterium parvum*, alter the reticulo- ıelial system, causing enhancement of :ytic clearance of particles and in- ı microbicidal activity (7). These im- nodulators also markedly enhance the :y of the host to inhibit tumor growth, ıe antitumor mechanism has been strated to involve activated macro- (8, 9). More recently, levamisole has emonstrated to enhance resistance to ıfection, although the mechanism re- poorly defined (2). The present stud- re undertaken to compare the anti- ctivities of several of these diverse ıomodulators in two experimental simplex virus models which were se- to provide a systemic infection (intra- ıeal route) and a local infection (in- nal route) that is characterized by direct nerve root spread to the central nervous system (10). Experiments were also designed to investigate whether the immunomodulators would be effective in mice with impaired host resistance.

Materials and methods. Mice. Female Swiss mice obtained from Simonsen Laboratories, Gilroy, Calif. were used for all experiments.

Drugs. Corynebacterium acnes (ATCC 11828) was grown as previously described (11), the organisms were washed, suspended at 15 mg/ml wet weight, heat-killed at 60° for 1 hr, and stored at 4°. A killed vaccine of *C. parvum*, containing 7 mg/ml wet weight of organisms, was received from Burroughs-Wellcome, Research Triangle, N.C. Pyran (lot XA 124-177) was purchased from Hercules, Inc., Wilmington, Del., suspended in phosphate-buffered saline (PBS), and brought into solution by the addition of NaOH to pH 7.0. A 2.5% solution of oyster glycogen type II (Sigma Chemical Co., St. Louis, Mo.) was prepared in PBS and stored at 4°. Levamisole (R12564, Janssen Research and Development Co., New Brunswick, N.J.), lot 75A15/786, was obtained courtesy of Dr. Gerald Fisher, Tripler Army Medical Center, Honolulu, Hawaii, and dissolved in PBS. Silica No. 12, particle size between 2 and 10 nm, was obtained courtesy of Dr. Benkert, Dorentruper Sand-und Thonwerke BmbH, Dorentrup, West Germany. For some experiments, silica, 325 mesh (Sargeant-Welch Co.) was prepared as described by Selgrade and Osborn (12).

Virus and Cells. A pool of herpes simplex virus type 2 (HSV-2), MS strain, was prepared in primary rabbit kidney cells as previously described (10), and titered 5×10^6 plaque-forming units (PFU) on secondary mouse embryo fibroblasts (MEF). Studies of HSV-2 pathogenesis were performed as previously described (10). Fetal lamb kid-

ıported by NIH research Grants AI-10217 and 93. P.S.M. is the recipient of USPHS Research Development Award AI 70863.

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ɔ 1977 by the Society for Experimental Biology and Medicine served.

ISSN 0037

ney cells were used for titration of HSV-2 in organs. All medium consisted of 10% inactivated fetal calf serum, Eagle's minimal essential medium with Earle's balanced salt solution (EMEM), 100 units/ml of penicillin, and 50 μg/ml of streptomycin.

Antiviral studies in vivo. Swiss mice were prophylactically treated with the immunomodulators according to the following regimens: *C. acnes*, 150 mg/kg ip, Day −14; *C. parvum*, 70 mg/kg ip, Day −14; pyran, 25 mg/kg iv, Day −1 or 75 mg/kg ip, Day −1; and levamisole 1 mg/kg ip, Day −1. Controls included mice inoculated ip with glycogen (0.2 ml, Day −5) or PBS (0.2 ml, Day −14).

Mice were challenged ip with approximately 2×10^5 PFU of virus, or intravaginally (i.vag.) with approximately 1×10^5 PFU as previously described (10). Experimental groups of animals usually consisted of at least 15 mice. Animals were checked daily for mortality. The mean survival time was calculated and compared with that in control groups by the Student *t* test. The mortality in drug-treated as compared with control groups was evaluated using the χ^2 test with Yates' correction factor.

Results. Comparison of immunomodulators against ip and i.vag. HSV-2 infection in adult mice. Prophylactic systemic treatment (iv) of mice with pyran or treatment with corynebacteria or pyran at the site of virus inoculation (ip) significantly protected mice against lethal systemic infection with HSV-2 (Table I). In contrast, treatment by the same regimens did not significantly alter mortality of a local genital HSV-2 infection of mice, in which the virus spreads by direct nerve root extension to the central nervous system (10). The mean survival time of intravaginally infected mice was significantly increased, however, by systemic treatment with pyran. The results are similar to previous findings (13, 14) which have demonstrated that iv treatment of young adult BALB/c mice with pyran can significantly decrease the mortality of mice infected intravenously or intravaginally, but that the activity against the vaginal infection was less effective than against the systemic infection. Treatment with levamisole or glycogen was ineffective against either infection. In another experiment levamisole was administered twice, on the day prior to and the day of ip HSV-2 infection; this regimen was also ineffective (data not shown).

Treatment of suckling rats with levamisole has been reported to increase resistance to HSV-2 infection (2), and experiments were thus designed to determine the effectiveness of immunomodulators in young mice. Suckling mice averaging 8 days of age were treated ip with pyran or levamisole and infected ip with HSV-2 24 hr later. Levamisole had no protective effect on the course of the disease, as measured either by a reduction in mortality or prolongation of survival time. Treatment with pyran produced a slight reduction in mortality from 100% in controls to 75% ($P < 0.05$), and prolonged the survival time of mice from 6.0 ± 0.2 days in the control group to 9.8 ± 0.6 days in the pyran-treated group ($P < 0.01$). An attempt was also made to determine whether levamisole, in conjunction with *C.*

TABLE I. COMPARISON OF IMMUNOMODULATOR-INDUCED RESISTANCE AGAINST HSV-2 INFECTION IN ADULT MICE.[a]

	Infection ip		Infection i. vag.		
Treatment	Dead/Total	Percent	Dead/Total	Percent	Mean survival time ± SE
PBS	15/15	100	9/15	60	7.7 ± 0.2
C. acnes	3/15*	20	13/16	81	8.5 ± 0.4
C. parvum	4/14*	29	14/15	93	8.8 ± 0.3
Pyran ip	7/15*	47	12/15	80	10.9 ± 0.9
Pyran iv	8/15*	53	8/14	57	10.6 ± 0.6*
Levamisole	11/15	73	12/15	80	9.2 ± 0.4
Glycogen	11/15	73	9/15	60	8.2 ± 0.3

[a] Mice were treated with the immunomodulators as indicated in Materials and methods, inoculated ip or i. vag. with HSV-2, and the mortality and mean survival time of the mice that died were calculated.

* $P < 0.05$.

eatment, could protect young mice HSV-2 infection. Treatment of suck- e with *C. acnes* alone was ineffective ıd it was possible that levamisole nt could enhance immunologic mat- processes. Treatment of 8-day-old th levamisole, *C. acnes* alone, or the ıtion of the two immunomodulators, r, had no protective effect on the ɔf disease in mice infected 14 days weeks old) with HSV-2. Prophylac- :ment of the 3-week old mice with 75 mg/kg ip, −24 hr) was similar to nt of suckling mice, causing a slight ɔn in mortality and an increased ırvival time from 8.2 ± 0.4 to 12.2 ays ($P < 0.05$).

: of silica treatment on HSV-2 infec- l immunomodulator-induced protec-

he antiviral action of the corynebac- pyran were mediated through mac- :s, less protection might be evident : depleted of macrophages. Silica nt, which alters a number of immu- responses, is toxic for macrophages nsiently depletes the macrophage ion (15, 20, 21). When differential were performed on peritoneal cells d 2–24 hr after ip silica treatment, it parent that the monocyte/macro- nd lymphocyte populations were de- while the polymorphonuclear cell ion was dramatically increased from to approximately 50% of the popu- Although there is considerable varia- :h the use of silica, treatment with f two different preparations of silica ip inoculation of HSV-2 consistently :d mortality to the disease at least as compared to control animals (Ta-

ncrease in mortality was reflected in ;e in the viral pathogenesis. When :re inoculated ip with 2×10^3 PFU , 20% mortality occurred in the un- control and 100% in the silica- group. Organs of groups of three om each group were obtained and for assay at intervals during the ɔf the disease. In silica-treated mice, ıs present in all of the visceral organs ıfter infection, with the highest titers ;pleen, kidney, and blood (Fig. 1).

TABLE II. EFFECT OF SILICA TREATMENT ON SYSTEMIC HSV-2 INFECTION.[a]

Experiments	$\log_{10}$ LD_{50}	
	Normal mice	Silica-treated mice
Experiment 1		
Sargeant-Welch silica	2.7	3.7
Dorentruper silica	2.7	3.7
Experiment 2		
Dorentruper silica	2.4	3.7

[a] Adult mice were treated ip with PBS, Sargeant-Welch silica (50 mg/kg), or Dorentruper silica (40 mg/kg) 2 hr prior to ip challenge with 10-fold dilutions of HSV-2, mortality was observed, and the LD_{50} doses per 0.2 ml of inoculum were calculated.

Virus persisted systemically until at least Day 4, at which time HSV-2 was present in both the spinal cord and brain stem. Progressive growth occurred in the central nervous system, with HSV-2 appearing in the cerebrum on Day 6. In the control group, low levels of virus were found sporadically in visceral organs, and virus did not appear in the central nervous system until Day 6. In untreated adult animals infected ip with a lethal dose (1×10^5 PFU) of HSV-2, virus also appears in the visceral organs late in the course of infection (Day 7) and primarily is in the spleen, lung, and kidney (Kern, unpublished observations).

The pathogenesis results indicated that it would not be appropriate to determine the protective effects of an immunomodulator in normal and silica-treated animals infected with the same dose of HSV-2, since the effective challenge in silica-treated mice would be greater than that in normal mice. Thus, mice were treated with the immunomodulators, then with PBS or silica, and challenged with 10-fold dilutions of HSV-2 in order to determine the effective LD_{50} in each group. Although silica treatment enhanced the LD_{50} of mice more than 100-fold, the immunomodulators were as effective in silica-treated mice as in normal mice (Table III). In fact, the LD_{50} of HSV-2 in *C. acnes*- or pyran-treated mice was almost the same in normal and macrophage-depleted mice.

Discussion. In this comparative study, adult mice were markedly protected from

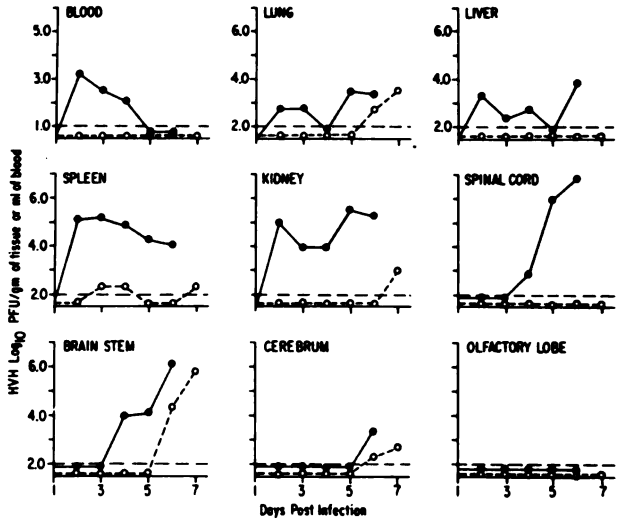

FIG. 1. The effect of silica treatment on the pathogenesis of herpes simplex virus (HVH) type 2 infection of mice. Virus titers in organs from silica-treated mice (●) or PBS-treated mice (○).

TABLE III. LACK OF EFFECT OF SILICA ON ACTIVITY OF C. ACNES OR PYRAN AGAINST HSV-2 Infection[a]

Immunomodulator treatment	PBS		Silica	
	$\log_{10}$ LD_{50}	Protection ($\log_{10}$)	$\log_{10}$ LD_{50}	Protection ($\log_{10}$)
PBS	2.3		4.7	
C. acnes	0.9	1.4	1.0	3.7
Pyran iv	1.4	0.9	1.7	3.0

[a] Groups of 15 mice each were treated ip with PBS or *C. acnes* (150 mg/kg) 14 days prior to, or with pyran (25 mg/kg iv) 1 day prior to virus challenge. Mice were treated with silica (40 mg/mouse ip) or PBS 2 hr prior to ip challenge with tenfold dilutions of HSV-2 ranging from $10^{-0.7}$ to $10^{-5.7}$.

intraperitoneal infection with HSV-2 by administration of pyran or killed corynebacteria, similar to data reported separately for *C. acnes* and pyran (13, 16). While both agents were effective against ip HSV-2 infection, only pyran significantly increased the survival time of mice infected intravaginally. It is of interest that prophylaxis with another immunomodulator, *Mycobacterium bovis* strain BCG, had no effect on mortality of mice from intravaginal HSV-2 infection (17). The results suggested that the antiviral effect of corynebacteria against HSV-2 might be exerted locally rather than systemically. Local administration of *C. parvum* appears to be more effective than does systemic administration against tumor growth (8), while the antitumor and antimicrobial action of pyran is expressed both locally and systemically (9). The differences between the two immunomodulators may be related to the induction by *C. parvum* of local granulomas with activated macrophages (8), while granuloma formation is not prominent after pyran administration (18).

Treatment of either adult or suckling mice with the immunomodulator levamisole was ineffective. These results are in contrast to those reported by Fisher *et al.* (2), who demonstrated marked protection of suckling 10-day-old rats from HSV-2 infection. Starr *et al.* (6) have also recently reported the ineffectiveness of levamisole, typhoid vaccine, brucella vaccine, or staphage lysate against HSV-2 infection in suckling mice. Only prophylactic BCG treatment was able to modify the survival rate of young mice. The mechanism of antiviral action of BCG and pyran in young mice remains to be defined.

Experiments were performed in an effort to determine whether activated macrophages were involved in the antiviral resistance produced by pyran and the corynebacteria. Zisman *et al.* (19) and DuBuy (20)

demonstrated that adult mice can be more susceptible to HSV infection if ıice are treated with silica, which is ively toxic for macrophages (21). The ıt experiments with silica established egree of enhanced susceptibility; the in silica-treated mice was consistently ısed 10-fold or more over that in con- Our results confirm and extend the vations of Zisman *et al.* (19) on patho- is of the infection. In silica-treated virus replication in the visceral organs ignificantly enhanced. The infection ssociated with a viremia, earlier seed- f the central nervous system, and a r survival time than in untreated mice. ugh silica treatment profoundly al- the course of the disease, animals re- g the immunomodulators pyran or *C.* were protected as effectively as nor- nice. These data would suggest that phages or other possible immunologic onents affected by silica (15, 21) are e critical determinant in the protective s of the immunomodulators. How- the immunomodulator treatments an influx of macrophages into the per- l cavity and an hepatosplenamegaly ated with increased reticuloendothe- pacity (7, 8), which could decrease the iveness of silica. Immunomodulators as *C. parvum* have also been demon- d to increase macrophage colony for- n from stem cells in the bone marrow If the immunomodulators can amplify sidual reserves of cells, it may be im- ble to deplete mice sufficiently of mac- ges.

ults of pathogenesis studies have sug- l that the antiviral effects of these im- modulators are exerted during the first s of the viral infection and do not in- enhancement of specific immunologic nses, but rather involve nonspecific re- ce factors (14, 16). Previous results pyran have demonstrated that the drug s effective in immunosuppressed mice ent in T-cell function as it was in nor- ıice (23). The current results indicate he pyran and *Corynebacterium* immu- dulators were also effective in mice edly deficient in host resistance func- due to silica treatment. Further studies on the mechanism of action of these substances are currently underway. It is clear that certain immunomodulators have the potential for ameliorating virus infections that are exacerbated as a result of immunosuppression.

Summary. Adult mice were protected from mortality after intraperitoneal infection with herpes simplex virus type 2 by prophylactic treatment with the immunomodulators, *Corynebacterium parvum, Corynebacterium acnes*, or pyran. Treatment with pyran, but not with the corynebacteria, also increased the survival time of mice after local genital (intravaginal) infection with the virus. Treatment with levamisole or glycogen was ineffective against either virus infection. Levamisole and *C. acnes* were also ineffective against herpes simplex virus infection in suckling and weanling mice, while pyran was slightly effective. Silica treatment, to suppress macrophage function *in vivo*, increased the susceptibility of mice to herpes simplex infection by 10- to 100-fold. The increased susceptibility induced by silica was associated with early, sustained viral replication in the visceral organs. Although silica treatment markedly suppressed host resistance to herpes virus, this treatment did not inhibit the antiviral activity of pyran or *C. acnes*.

The authors appreciate the excellent technical assistance of James T. Richards.

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Received November 3, 1976. P.S.E.B.M. 1977, Vo 154.

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